STUDIES ON THE CHARACTERISATION AND MODULATION OF CD4+ T LYMPHOCYTE RESPONSES TO ANTIGENS THAT INDUCE RESPIRATORY TRACT INFLAMMATION

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1999

CONTENTS

To Deborah, Rachel and Eleanor

DECLARATION

The scientific papers and reviews listed below that were published up to March 1988 were submitted for consideration for membership of the Royal College of Pathologists.

The written work submitted here has been generated by research groups and the contribution made by the candidate to each submission as regards laboratory work (L), writing of the paper (W) and intellectual input (I) is indicated as a percentage.

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ABSTRACT

Regulation of the heterogeneous cell types and mediators that constitute the immune system is achieved, in part, by the activity of CD4+ T lymphocytes. Their effector functions are induced following ligation of antigen specific receptors expressed on the cell surface of the CD4+ T cells by peptides bound to major histocompatibility complex class II proteins, in conjunction with costimulatory signals provided by antigen presenting cells. Qualitative and quantitative differences in CD4+ T cell effector functions allow the immune system to maintain homeostasis through its ability both to induce tolerance and promote productive immunity. The failure of CD4+ T cells to recognise antigen or stimulate inappropriate immune responses also influences the outcome of immunity, the resolution of inflammation and, therefore, in some circumstances may compromise the host.

The research reported here investigates the biology and regulation of CD4+ T cell responses to antigens capable of inducing inflammation of the respiratory tract. Initially, T cell recognition of the respiratory pathogens, influenza virus and mycobacteria, is described. CD4+ T cells also contribute to chronic airway inflammation that occurs in susceptible individuals when they are exposed to environmental allergens and the characteristics of allergen reactive T cells are defined for responses to house dust mite derived allergens. Molecular studies complete the research on T cell antigen recognition and provide the scientific basis for the development of both *in vitro* and *in vivo* experimental models of CD4+ T cell targeted immunotherapy which are then described. The observations from this analysis has potential practical applications in the regulation of allergic inflammation.

In summary, the research reported here explores the biology of CD4+ T cells specific for different antigens associated with respiratory inflammation and their potential to act as targets in the induction of protective immunity or the modulation of unwanted immune responses.

CHAPTER 1

CD4+ T CELL ANTIGEN RECOGNITION

1.1 INTRODUCTION

CD4+ T lymphocytes regulate many aspects of innate immunity, such as macrophage activation, as well as those of the adaptive immune system, for example the induction of cytotoxic T cells and the regulation of antibody synthesis. The research described in this thesis focuses on the characterisation and regulation of CD4+ T cells activated by stimulation with different antigens that can lead to inflammation of the airways. As examples of respiratory pathogens, capable of inducing either acute or chronic inflammation, CD4+ T cell responses to influenza virus and mycobacteria were investigated. These findings are compared to the CD4+ T cell repertoire that is induced following the inhalation of non-pathogenic environmental antigens, such as allergens derived from house dust mite (HDM). HDM is the most common source of aeroallergens and causes symptoms that range from atopic dermatitis and rhinitis to asthma. Therefore, in light of its clinical importance HDM allergy was selected for investigation as an example of unwanted chronic immunity generated by inhaled non-pathogenic antigens.

Collectively influenza, mycobacterial infection and HDM induced allergic disease, all of which can effect the respiratory tract, constitute a major global health problem. Through immune regulation in the form of vaccination the severity of the symptoms caused by these agents may be reduced or prevented. However, current vaccines for influenza and mycobacteria need improvement and as an initial step in the design of new vaccines detailed information on the immune responses against these pathogens is required. CD4+ T cells play an important role in immunity to influenza and mycobacterial infection, therefore, detailed analysis of the T cell repertoire in both individuals with disease and those who are protected will provide valuable information. As regards HDM induced airway inflammation CD4+ T (Th2) cells promote specific IgE synthesis and the differentiation and activation of eosinophils. Thus, the aim of specific vaccination in this situation is to inhibit the function of Th2 cells that promote allergic inflammatory responses. Therefore, characterisation of these cells may contribute to the development of improved allergen immunotherapy.

In the first section of this chapter T cell recognition of influenza, mycobacteria and HDM derived antigens in health and disease is investigated with respect to antigen (sections 1.2.1 to 1.2.3) and MHC class II restriction (section 1.3) specificity. This is followed in section 1.4 by publications reporting experiments which were designed to explore the molecular interactions formed between T cell antigen receptor (TCR), MHC class II molecules and antigenic peptides or superantigens. The information obtained from the results of these experiments form, in part, the scientific basis of the different approaches to immunotherapy specifically designed to target CD4+ T cells (chapter 2).

1.2 ANALYSIS OF THE ANTIGEN SPECIFICITY OF T CELL RESPONSES

1.2.1 INFLUENZA VIRAL ANTIGENS

1.2.1.1 Introduction

Influenza virus induces acute infection that results in the recruitment and expansion of both viral specific CD4+ and CD8+ T cells, as well as the production of neutralising antibodies, which are predominantly directed against the cell surface glycoprotein, haemagglutinin (HA). CD8+ T cells, which tend to target cross reactive antigens, such as matrix protein (MP) and nucleoprotein (NP), also contribute to protection against and recovery from influenza infection. However, it appears that humoral immunity may play the more important role in protection and this is illustrated in experimental models of influenza virus infection where it has been demonstrated that passively transferred anti-HA antibodies alone can mediate protection. Therefore, in the analysis of the influenza virus specific CD4+ T cell repertoire reported here recognition of HA was of particular interest. These studies were extended to determine the effects of both natural infection and subunit vaccination on the specificity of the HA reactive T cell repertoire. Although much emphasis has been placed on HA specific responses CD4+ T cell recognition of neuraminidase (NA) and the core proteins (MP and NP) was also investigated .

Lamb JR, Eckels DD, Lake P, Johnson AH, Hartzman RJ, Woody JN: Antigen specific human T lymphocyte clones: induction, antigen specificity, and MHC restriction of influenza virus-immune clones. *J Immunol* 1982, **128**:233-238.

Lamb JR, Eckels DD, Phelan M, Lake P, Woody JN: Antigen-specific human T lymphocyte clones: viral antigen specificity of influenza virus-immune clones. *J Immunol* 1982, **128**:1428-1432.

Lamb JR, Eckels DD, Lake P, Woody JN, Green N: Human T-cell clones recognize chemically synthesized peptides of influenza haemagglutinin. *Nature* 1982, **300**:66-69.

Gelder CM, Welsh K, Faith A, Lamb JR, Askonas BA: Human $CD4^+$ T-cell repertoire responses to influenza A haemagglutinin after recent natural infection. *J Virol* 1995, **69**:7497-7506.

Gelder CM, Lamb JR, Askonas BA: Human $CD4^+$ T-cell recognition of influenza virus A haemagglutinin after subunit vaccination. *J Virol* 1996, **70**:4787-4790.

1.2.1.2 Summary

Analysis of the antigen specificity of human CD4+ T cell responses by clonal analysis in an adult following recent infection with influenza A revealed that cells reactive with HA, NA, MP and NP were present in the peripheral repertoire. Of the 11 T cell clones investigated 5 were specific for NA, 4 for MP, one was specific for NP and one for HA. The HA specific T cells were subtype specific responding to all the H3N2 influenza viruses tested, whereas the NA specific cells recognised H3N2 and H2N2 influenza viruses. Analysis of the MP and NP reactive T cell clones using different viral subtypes demonstrated that they were broadly cross reactive amongst the influenza A viruses.

In order to explore the HA reactive human CD4+ T cell repertoire in greater depth T cell lines and clones were induced with affinity purified H3 and their specificity analysed using different viruses representative of the different viral subtypes together with synthetic peptides. The results of these experiments revealed T cell epitopes within residues 1-38, 105-140, 200-228 and 306-318 of HA1 of which 306-318 was immunodominant at least for the HLA-DR1, 3 haplotype. This latter region is conserved for the H3 subtypes. Only a single T cell clones was identified that was uniquely specific for H3 of A/Texas/1/77 the inducing antigen. Other T cells revealed varying levels of cross reactivity.

In more recent studies CD4+ T cell recognition of HA from A/Beijing/32/92 was examined in 3 groups of unrelated adults of different HLA haplotypes. Those in group 1 were naturally infected with A/Beijing. Group 2 comprised of subjects with no history of influenza virus infection or vaccination 4 years prior to the study. The final experimental group contained individuals that were vaccinated with trivalent influenza subunit vaccine containing HA from A/Beijing/32/92. Using synthetic peptides covering the entire HA1 and HA2 subunits the specificity of CD4+ T cell responses were investigated. Following natural infection T cell responses are directed towards highly conserved regions, with all subjects responding to epitopes within sequences 295-328 and 407-442. Vaccination boosted the response to conserved epitopes and promoted the expansion of cross reactive T cells. Although T cell recognition of HA epitopes was strongly influenced by HLA class II the affinity of the binding of particular peptides did not necessarily mean that these specificites would dominant the T cell response.

1.2.1.3 Related publications

Lamb JR, Woody JN, Hartzman RJ, Eckels DD: *In vitro* influenza virus specific antibody production in man: antigen specific and HLA restricted induction of helper activity mediated by cloned human T lymphocytes. *J Immunol* 1982, **129**:1465-1470.

Lamb JR, Green N: Analysis of the antigen specificity of influenza haemagglutinin immune human T lymphocyte clones: identification of an immunodominant region for T cells. *Immunology* 1983, **50**:659-666.

Jones C, Lake RA, Lamb JR, Faith A: Degeneracy of TCR recognition of an influenza virus haemagglutinin epitope restricted by HLA-DQ and -DR class II molecules. *Eur J Immunol* 1994, **24**:1137-1142.

1.2.2 MYCOBACTERIAL ANTIGENS

1.2.2.1 Introduction

In contrast to influenza, infection with mycobacteria may result in chronic inflammation that may fail to resolve and one of the primary target organs affected is the lung. Evidence suggests that immunological protection against mycobacteria is dependent on the effector function of specific CD4+

T cells, representative of the Th1 functional phenotype, perhaps with some contribution from CD8+ T cells. However, it seems that humoral immunity offers minimal protection if any at all. Therefore, in view of the quanlitative differences in protective immunity to influenza and mycobacterial infection it was of interest to contrast the nature of CD4+ T cell responses to these pathogens as regards recognition of specific and/or cross reactive determinants in their target antigens.

The prinicipal aims of the studies on CD4+ T cell recognition of mycobacterial components (section 1.2.2) were two fold. Firstly, to determine if T cells could be identified that recognised antigenic determinants that are expressed in *Mycobacterium tuberculosis* and not *Mycobacterium bovis* (BCG). Secondly, to explore from the analysis of the pattern of antigen specific T cell recognition in family contacts and patients with mycobacterial infection if there was any indication of those antigens that may be associated with protective immunity or susceptibility to disease. Collectively, this information might be of value in the design of skin test reagents to distinguish BCG vaccinated individuals from those with active disease, such as tuberculosis and contribute to the development of subunit vaccines.

A major hurdle in the analysis of the antigen specificity of T cells reactive with mycobacterial antigens has been the restricted availability of antigens in a purified form. The number of proteins in *M. tuberculosis* that are potential candidates of T cell recognition is extensive. While the use of different strains of mycobacteria, affinity purified antigens, recombinant proteins and peptides synthesized from predicted protein sequences can provide some information it is limited and allows only a proportion of the T cell repertoire to be investigated. In order to circumvent this problem a solid-phase an assay using nitrocellulose immunoblots was developed to screen fractionated mycobacteria and recombinant libraries. The diversity of CD4+ T cell responses to mycobacterial antigens was explored and the immunodominance of cross-reactive epitopes established. In addition, it was observed that several of the antigens that were targets of T cell recognition were stress proteins, which have human homologues. Cross reactive immunity to these antigens may provide a link between infectious and autoimmune diseases.

Young DB, Kent L, Rees ADM, Lamb JR, Ivanyi J: Immunological activity of a 38-kilodalton protein purified from *Mycobacterium tuberculosis*. *Infect Immun* 1986, **54**:177-183.

Lamb JR, Ivanyi J, Rees ADM, Rothbard JB, Howland K, Young RA, Young DB: Mapping of T cell epitopes using recombinant antigens and synthetic peptides. *EMBO J* 1987, **6**:1245-1249.

Lamb JR, Young DB: A novel approach to the identification of T-cell epitopes in *Mycobacterium tuberculosis* using human T-lymphocyte clones. *Immunology* 1987, **60**:1-5.

Mendez-Samperio P, Lamb JR, Bothamley G, Stanley P, Ellis C, Ivanyi J: Molecular study of the T cell repertoire in family contacts and patients with leprosy. *J Immunol* 1989, **142**:3599-3604.

Young DB, Ivanyi J, Cox JH, Lamb JR: The 65kDa antigen of mycobacteria - a common bacterial protein? *Immunol Today* 1987, **8**:215-219.

Lamb JR, Bal V, Mendez-Samperio P, Mehlert A, So A, Rothbard JB, Jindal S, Young RA, Young DB: Stress proteins may provide a link between the immune response to infection and autoimmunity. *Int Immunol* 1989, **1**:191-197.

1.2.2.2 Summary

From analysis of polyclonal T cell populations and T cell clones it appeared that the major component of the human CD4+ T cell repertoire was cross reactive recognising antigens that are present in *M. tuberculosis*, *M. bovis* BCG and other strains of mycobacteria. Where possible, T cell recognition was further examined at the epitope level using the combination of truncated recombinant proteins and synthetic peptides. Several of the target antigens of T cell responses induced by mycobacteria are common bacterial proteins, for example the 65 kDa heat shock protein. There are vertebrate homologues of bacterial heat shock proteins. Thus, interest was generated in the possiblity that expression of human heat shock proteins, following trauma or cell death, may reactivate T cells initially primed with mycobacterial heat shock proteins. A theoretical consequence of this T cell cross reactivity is

the potential to induce or exacerbate autoimmune diseases, such as rheumatoid arthritis. However, rather than being the direct cause of specific autoimmune immune diseases it is likely that these cross reactive immune responses are present as a non-specific component characteristic of chronic inflammation in general.

Despite the molecular and serological definition of a variety of mycobacterial proteins, many remain that are uncharacterised and could be targets of the T cell response. In order to investigate this component of the repertoire mycobacterial antigens were fractionated, immunoblotted and used in solid-phase assays to analyse the antigen specificity of T cell responses. Using this approach an antigen in the molecular weight region of 52-55 kDa, which was distinct from known monoclonal antibody defined antigens, was identified as a T cell target. The antigen was present in *M. tuberculosis* but not *M.bovis* BCG. This technique was applied to the analysis of T cell repertoire reactive with *M. leprae* and *M. tuberculosis* in family contacts and patients with leprosy and provided a lead for the identification of immunodominant antigens. Although the pattern of T cell recognition was complex and influenced by HLA haplotypes, again there was evidence to suggest that distinct epitopes present in different strains of mycobacteria are recognised by T cells. Nevertheless, the major component of the T cell repertoire in the patients and control subjects was cross-reactive. With technical advances the molecular characterisation of strain specific T epitopes is becoming easier to realise and, therefore, more candidate vaccines will become available for evaluation.

1.2.2.3 Related publications

Collins FM, Lamb JR, Young DB: Biological activity of protein antigens isolated from *Mycobacterium tuberculosis* culture fitrate. *Infect Immun* 1988, **56**:1260-1266.

Rees A, Praputpittaya K, Scoging A, Dobson N, Young D, Ivanyi J, Lamb JR: T cell activation by anti-idiotypic antibody: evidence for the internal image. *Immunology* 1987, **60**:389-393.

Rees A, Scoging A, Dobson, N, Praputpittaya K, Young D, Ivanyi J, Lamb JR: T cell activation by anti-idiotypic antibody: mechanism of interaction with antigen reactive T cells. *Eur J Immunol* 1987, **17**:197-201.

Lamb JR, O'Hehir RE, Young DB: The use of nitrocellulose immunoblots for the analysis of antigen recognition by T lymphocytes. *J Immunol Methods* 1988, **110**:1-10.

Brett SJ, Lamb JR, Cox JH, Rothbard JB, Mehlert A, van Embden J, Ivanyi J: Differential pattern of T cell recognition of the 65kDa mycobacterial antigen following immunization with the whole protein or peptides. *Eur J Immunol* 1989, **19**:1303-1310.

Lamb JR, Young DB: T cell recognition of stress proteins: a link between infectious and autoimmune disease. *Mol Biol Med* 1990, **7**:311-321.

D'Souza S, Levin M, Faith A, Yssel H, Bennett B, Lake RA, Brown IN, Lamb JR: Diminished interferon-gamma production in disseminated *Mycobacterium avium* complex infections. *Clin Exp Immunol* 1996, **103**:35-39.

1.2.3 HOUSE DUST MITE DERIVED ALLERGENS

1.2.3.1 Introduction

The inhalation of non-pathogenic environmental antigens has opposing immunological outcomes in individuals who are susceptible to allergen induced respiratory inflammation and those who are not. In the latter population inhaled antigen does not prime productive immunity but on the contrary induces non-responsiveness. In those individuals who suffer from allergic disease of the airways a hallmark of inflammation is the presence of CD4+ T cells expressing a Th2-dominant cytokine profile, the infiltration of eosinophils and, in many instances, the production of specific IgE antibodies. It is believed that eosinophils contribute to airway hyperresponsiveness in asthma through epithelial damage mediated by basic granule proteins, and that similar mechanisms may also contribute to upper airway inflammation in rhinitis. The induction of IgE and the recruitment of eosinophils are mediated by CD4+ T cells. A primary step in the development of CD4+ targeted immunotherapy for HDM induced allergy is to characterise the allergen specific T cell repertoire. Here, the specificity and biological activity of T cells reactive with HDM derived allergens were investigated for polyclonal and monoclonal cell populations as an example of chronic inflammatory disease.

O'Hehir RE, Young DB, Kay AB, Lamb JR: Cloned human T lymphocytes reactive with *Dermatophagoides farinae* (house dust mite): a comparison of T- and B- cell antigen recognition. *Immunology* 1987, **62**:635-640.

O'Hehir RE, Bal V, Quint D, Moqbel R, Kay AB, Zanders ED, Lamb JR: An *in vitro* model of allergen dependent IgE synthesis by human B cells: comparison of the response of an atopic and non-atopic individual to *Dermatophagoides* spp. (house dust mite). *Immunology* 1989, **66**:499-504.

Wedderburn LR, O'Hehir RE, Hewitt CRA, Lamb JR, Owen MJ: *In vivo* clonal dominance and limited T-cell receptor usage in human $CD4^+$ T-cell recognition of house dust mite allergens. *Proc Natl Acad Sci USA* 1993, **90**:8214-8218.

O'Hehir RE, Verhoef A, Panagiotopoulou E, Keswani S, Thomas WR, Lamb JR: Analysis of human T cell responses to the group II allergen of *Dermatophagoides* spp. (house dust mite): localization of major antigenic sites. *J Allergy Clin Immunol* 1993, **92**:105-113.

Kristensen N, Hoyne GF, Hayball JD, Hetzel C, Bourne T, Lamb JR: Induction of T cell responses to the invariant chain derived CLIP peptide in mice immunized with the group 1 allergen of house dust mite. *Int Immunol* 1996, **8**:1091-1098.

van Neerven RJJ, Ebner C, Yssel H, Kapsenberg ML, Lamb JR: T-lymphocyte responses to allergens: epitope-specificity and clinical relevance. *Immunol Today* 1996, **17**:256-532.

1.2.3.2 Summary

Analysis of antigen specificity revealed that human CD4+ T cell responses to influenza virus and mycobacteria are predominantly cross reactive amongst different strains of the respective pathogens. Cross reactivity may occur as the result of repeated exposure leading to the selection of particular epitopes that may be more abundant, processed more efficiently or bind to MHC class II with higher affinity. Alternatively, it may arise from recognition of common antigens that are present in different microorganisms.

The situation is different for T cell responses to HDM derived allergens in allergic individuals. Although there is extensive cross reactivity of antibodies for the different species of HDM, the T cell repertoire was specific for only the inducing species despite extensive sequence homology. The studies were extended and epitope maps were generated for T cell responses to the group 1 (Der p 1) and 2 (Der p 2) allergens of *Dermatophagoides pteronyssinus*. From the results of experiments using overlapping peptides to examine polyclonal T cell responses to Der p 2 it appeared that for individual donors multiple epitopes were recognised, although selected regions of the protein were immunodominant. There was no evidence to suggest that particular epitopes were associated with T cell responses observed only in atopic individuals and it appeared that epitope recognition reflected MHC haplotype rather than disease status. Furthermore, the functional phenotype of the T cell repertoire in atopics was that of Th2/Th0 cells and for non-atopics, who also are capable of mounting T cell responses to HDM derived allergens, the Th1/Th0 phenotype was dominant. Human CD4+ T cell responses to Der p 1 were directed primarily towards epitopes located within the region 100-130, although other amino and carboxyl terminal regions were also immunogenic. These observations have bearing on the potential of peptide based immunotherapy for HDM induced allergic diseases at the population level. However, analysis of peripheral TCR repertoire within the same HDM atopic individual over a period spanning 6 years revealed that Vα8 and Vβ3 sequences were dominant and that identical junctional regions were present implying that chronic allergen exposure selects for long-lived T cell clones *in vivo*.

H-2^b (C57BL/6J) mice are high responders to Der p 1 and, although, the region 110-130 contains the immunodominant determinants, responses to minor epitopes (residues 15-29, 81-102 and 197-212) were also observed. The ability of Der p 1 to induce CD4+ T cells that proliferate to residues 15-29 (p1, 15-29) of Der p 1 and to the murine MHC class II-associated invariant chain derived peptide (CLIP) was demonstrated. In the absence of p 1, 15-29 or exogenously added CLIP, T cells from Der p 1 primed mice could be expanded on wild type H-2^b spleen cells and they retained specificity for both p 1, 15-25 and CLIP. This finding implies that naturally presented self peptides may maintain T cell memory to foreign antigens and in the case of HDM allergen contribute to the chronicity of the disease.

Chronic exposure to HDM induces a long-lived stable population of CD4+T cells that is largely species specific and not cross reactive which is in contrast to the human T cell repertoires reactive with either influenza or mycobacterial antigens.

1.2.3.3.Related publications

O'Hehir RE, Garman RD, Greenstein JL, Lamb JR: The specificity and regulation of T cell responsiveness to allergens. *Ann Rev Immunol* 1991, **9**:76-95.

1.3 ANALYSIS OF RESTRICTION SPECIFICITY BY MAJOR HISTOCOMPATIBILITY COMPLEX CLASS II PROTEINS

1.3.1 Introduction

Activation of CD4+ T cells depends upon the interaction of TCR with complexes formed by peptides bound to class II MHC proteins expressed on the surface of APCs. The recognition of these complexes is both restricted to particular MHC class II molecules and specific for a limited number of pepides. The experiments described here were designed to examine the restriction specificity of human CD4+ T cell recognition of influenza, mycobateria and HDM derived antigens. The practical application of this information would be in the development of subunit vaccines.

Eckels DD, Sell TW, Bronson SR, Johnson AH, Hartzman RJ, Lamb JR: Human helper T-cell clones that recognize different influenza haemagglutinin determinants are restricted by different HLA-D region epitopes. *Immunogenetics* 1984, **19**:409-423.

Eckels DD, Lake P, Lamb JR, Johnson AH, Shaw S, Woody JN, Hartzman RJ: *SB*-restricted presentation of influenza and herpes simplex virus antigens to human T-lymphocyte clones. *Nature* 1983, **301**:716-718.

Austin P, Trowsdale J, Rudd C, Bodmer W, Feldmann M, Lamb JR: Functional expression of *HLA-DP* genes transfected into mouse fibroblasts. *Nature* 1985, **313**:61-64.

Gelder C, Davenport M, Barnardo M, Lamb J, Askonas B, Hill A, Welsh K: Six unrelated HLA-DR matched adults recognize identical $CD4^+$ T cell epitopes from influenza A haemagglutinin that are not simply peptides with high HLA-DR binding affinities. *Int Immunol* 1998, **10**:211-222.

Lamb JR, Rees ADM, Bal V, Ikeda H, Wilkinson D, de Vries RRP, Rothbard JB: Prediction and identification of an HLA-DR-restricted T cell determinants in the 19-kDa protein of *Mycobacterium tuberculosis. Eur J Immunol* 1988, **18**:973-976.

Higgins JA, Thorpe C, Hayball JD, O'Hehir RE, Lamb JR: Overlapping T-cell epitopes in the group I allergen of *Dermatophagoides* species restricted by HLA-DP and HLA-DR class II molecules. *J Allergy Clin Immunol* 1994, **93**:891-899.

O'Hehir RE, Mach B, Berte C, Tiercy J-M, Bal V, Greenlaw R, Trowsdale J, Lechler R, Lamb JR: Direct evidence for a functional role of HLA-DRB1 and HLA-DRB3 gene products in the recognition of *Dermatophagoides* spp. (house dust mite) by helper T lymphocytes. *Int Immunol* 1990, **2**:885-892.

Verhoef A, Higgins JA, Thorpe C, Marsh SGE, Hayball JD, Lamb JR, O'Hehir RE: Clonal analysis of the atopic immune response to the group 2 allergen of *Dermatophagoides* spp: identification of HLA-DR and -DQ restricted T cell epitopes. *Int Immunol* 1993, **5**:1589-1599.

1.3.2. Summary

For T cell responses to influenza it was demonstrated that DRB1 gene products were the most frequently used restriction elements in the recognition of HA, NA, MP and NP. Similarly CD4+ T cell responses to mycobacterial antigens were restricted most commonly by HLA-DR molecules. Nevertheless, rare examples of influenza specific T cells restricted by DQ and DP were recorded. Peptide/ MHC class II protein interactions have a strong influence on the specificity of T cell responses. However, by analysing in parallel the class II binding affinity of HA peptides with immunodominance at the level of T cell proliferation it was apparent that there is not always a direct relationship. These findings suggest that antigen

processing, intracellular competition for HLA binding, the TCR repertoire and perhaps the level of antigen exposure may all determine immunodominance.

Extensive heterogeneity in the HLA class II restriction elements used by T cells for the recognition of HDM derived allergens was observed. In addition to DRB1, it was apparent that DRB3 gene products were also functional. Likewise, HDM specific T cell recognition restricted by HLA-DP and -DQ molecules was observed. Furthermore, there were examples of T cell restriction of a single T cell epitope by either HLA-DR or -DQ in the same individual. These observations may help to explain why it has proven difficult to establish HLA class II disease associations for HDM allergy. However, the practical advantage of promiscuity of HDM peptide binding when paralleled with immunodominance of the T cell response is in the design of subunit (peptide) vaccines.

1.3.3 Related publications

Eckels DD, Lamb JR, Lake P, Woody JN, Johnson AH, Hartzmann RJ: Antigen-specific human T-lymphocyte clones: Genetic restriction of influenza virus-specific responses to HLA-D region genes. *Human Immunol* 1982, **4**:313-324

O'Hehir RE, Eckels DD, Frew AJ, Kay AB, Lamb JR: MHC class II restriction specificity of cloned human T lymphocytes reactive with *Dermatophagoides farinae* (house dust mite). *Immunology* 1988, **64**:627-631.

1.4 MOLECULAR INTERACTIONS BETWEEN TCR, MHC CLASS II AND PEPTIDE OR SUPERANTIGEN MOLECULES

1.4.1 Introduction

Knowledge of the structural basis of the interactions formed between TCR, MHC class II, peptide or superantigen molecules has the potential to facilitate the enhancement, abrogation or selective modulation of T cell activation and effector function. This premise underlies the experiments described in the publications included in this section of the thesis. Much of the work reported here has focused on the interaction of the influenza HA peptide 306-318 with HLA-DRB1*0101 (DR1) which is recognised by the CD4+ T cell clone HA1.7. By the introduction of structural changes in the peptide ligand through amino acid substitutions and in MHC class II and TCR by site directed mutagenesis the nature of the interactions between these molecules has been

investigated. The subsequent resolution of the three dimensional structure of DR1/306-318 and DR1/SEB complexes by crystallography has helped in the interpretation of the results reported here which were derived from the combination of functional and cell surface binding assays.

Rothbard JB, Lechler R, Howland K, Bal V, Eckels D, Sekaly R, Long E, Taylor W, Lamb JR: Structural model of HLA-DR1 restricted T cell antigen recognition. *Cell* 1988, **52**:515-523.

Rothbard JB, Busch R, Howland K, Bal V, Fenton C, Taylor W, Lamb JR: Structural analysis of a peptide - HLA class II complex: identification of critical interactions for its formation and recognition by T cell receptor. *Int Immunol* 1989, **1**:479-486.

Busch R, Hill CM, Hayball J, Lamb JR, Rothbard JB: Effect of natural polymorphism at position 86 of HLA-DR β1 chain on peptide binding. *J Immunol* 1991, **147**:1292-1298.

Wedderburn LR, Searle SJM, Rees AR, Lamb JR, Owen MJ: Mapping T cell recognition: the identification of a T cell receptor residue critical to the specific interaction with an influenza haemagglutinin peptide. *Eur J Immunol* 1995, **25**:1654-1662.

Seth AH, Stern LJ, Ottenhof THM, Engel I, Owen MJ, Lamb JR, Klausner RD, Wiley DC: Binary and ternary complexes between T-cell receptor, class II MHC and superantigen *in vitro*. *Nature*. 1994, **369**:324-327.

1.4.2 Summary

The first approach used to probe the interactions between peptide and MHC class II molecules was based on analysing the effect of structural modification of the peptide ligand. In brief, this was achieved in several ways. Firstly, by synthesizing peptides truncated from the amino and carboxyl terminal ends. Secondly, by generating hybrid peptides formed by exchanging residues between two DR1 restricted epitopes (HA and MP) and determing the effects on the proliferative response of MP and HA specific T cell clones. Thirdly, by introducing single amino acid substitutions, radical and conservative, sequentially at each position and investigating the effect on

the ability of the peptide analogues both to bind to cell surface expressed MHC class II molecules and to induce T cell proliferation.

The existence of natural polymorphisms affecting a single or limited number of amino acids at known locations within MHC class II proteins, for example subtypes of DR4, allowed the role of residues at defined positions in T cell recognition to be investigated. Alternatively, substitutions can be made at selected residues by site directed mutagenesis and the mutant MHC class II molecules expressed in murine fibroblasts and their function determined. It is also possible to mutate residues at selected positions in the α and β chains of TCR and evaluate whether or not those changes abrogate or enhance antigen recognition. The TCR-α chain of cloned T cells reactive with the HA peptide 307-319 was mutated with E at position 94 substitued by A or K and both of these changes resulted in a loss antigen recognition. From molecular modelling it was predicted that TCR-α E94 would contact 316K in the HA peptide, however peptide analogues in which the residue at this position had been substituted failed to restore responsiveness. Thus, using a combination of technical approaches, it was possible to define residues within peptide, MHC class II and TCR molecules that influence antigen recognition.

Superantigens are presented by MHC class II proteins and although their recognition by T cells exhibits some degree of allele specificity they are not genetically restricted in the same way as conventional antigenic peptides. They stimulate T cells in an oligoclonal manner that is dependent on the expression of particular variable region gene elements in the TCR-β chain (Vβ). The possibility that T cell responses in certain diseases are associated with the expansion of particular TCR-Vβ families has attracted much attention. The potential to specifically regulate such "disease associated" T cell populations prompted the analysis of superantigen interactions with TCR and class II MHC proteins. The principal finding of this analysis was that unlike conventional peptides superantigens can form complexes with TCR in both the presence and absence of class II MHC molecules.

1.4.3 Related publications

Lechler RI, Bal V, Rothbard JB, Germain RN, Sekaly R, Long EO, Lamb JR: Structural and functional studies of HLA-DR restricted antigen recognition by human helper T lymphocyte clones using transfected murine cell lines. *J Immunol* 1988, **141:**3003-3009.

Cox JH, Ivanyi J, Young DB, Lamb JR, Syred AD, Francis MJ: Orientation of epitopes influences the immunogenicity of synthetic peptides dimers. *Eur J Immunol* 1989, **18**:2015-2019.

Rothbard JB, Busch R, Bal V, Trowsdale J, Lechler RI, Lamb JR: Reversal of HLA restriction by a point mutation in an antigenic peptide. *Int Immunol* 1989, **1**:487-495.

Buelow R, O'Hehir RE, Schreifels R, Kummerehl TJ, Riley G, Lamb JR: Localisation of the immunological activity in the superantigen staphylococcal enterotoxin B using truncated recombinant fusion proteins. *J Immunol* 1992, **148**:1-6.

Hayball JD, Robinson JH, O'Hehir RE, Verhoef A, Lamb JR, Lake RA: Identification of two binding sites in staphylococcal enterotoxin B that confer specificity for T cell receptor Vβ gene products. *Int Immunol* 1994, **6**:199-215.

CHAPTER 2

MODULATION OF CD4+ T RESPONSES

2.1 INTRODUCTION

In this chapter the focus is on CD4+ T cell targeted immunotherapy in the regulation of unwanted immune responses to inhaled non-pathogenic environmental antigens. Antigen independent adhesion between APCs and T cells is the primary event in CD4+ T cell activation and, therefore, the ability to disrupt this union may facilitate the inhibition of T cell responses. In the first section of the chapter experiments are performed to determine if the activation of HDM reactive Th0, Th1 and Th2 cells exhibit the same dependence on the functional activity provided by LFA-1 and CD4.

The initial interaction between T cells and APCs is followed by ligation of the TCR by complexes of antigenic peptides bound to class II MHC molecules expressed on the surface of APCs. Antigenic peptides are generated as the result of the processing of intact proteins, nevertheless, soluble peptides are able to bind directly to a small fraction of empty class II MHC molecules present on the cell surface. In principle, this subset of class II molecules is a possible target for immunotherapy since they can be used to present peptides delivered exogenously. Non-stimulatory peptides could potentially be used as antagonists to compete with the binding of "disease associated" T cell epitopes to cell surface class II MHC on the APCs. The potential of peptide competition at the level of antigen presentation to inhibit polyclonal and and clonal T cell responses to HDM derived allergens was investigated.

Subtle structural changes in antigenic peptides by the introduction of conservative amino acid substitutions can result in the generation of ligands which act as partial agonists. Peptides analogues with these characteristics are termed altered TCR ligands and, through their ability to differentially signal via the TCR, are capable of inducing selective T cell effector functions. The effects of altered TCR ligands on proliferation and cytokine production by HDM specific T cells was the principle focus of section 2.3.2 in chapter 2.

Ligation of the TCR by MHC class II/peptide complexes alone is insufficient to drive clonal expansion and additional costimulatory signals are required. To date it appears that the interaction of the B7 family of ligands (CD80 and CD86) with their receptors CD28 and CTLA-4 provides

costimulation. The presentation of antigen in the absence of costimulation leads to the induction of anergy and here an experimental model for the analysis of T cell anergy *in vitro* in human CD4+ T cells is described. The principal observation is that following exposure to a supraoptimal concentration of peptide in the absence of APCs T cells fail to respond to antigenic restimulation but remain responsive to exogenous IL-2. This basic finding is extended and questions are addressed which include the influence of antigen structure on tolerance induction as well as transcriptional regulation and phenotypic modulation in anergic T cells. The information that can be obtained from *in vitro* models has obvious limitations, therefore, the ability of allergen derivatives to modulate specific immune responsiveness was analysed *in vivo* in a mouse model of HDM induced immunity. The observation that inhalation of the immunodominant peptide of Der p 1 induces tolerance that can be transferred by CD4+ T cells prompted the analysis of regulatory (inhibitory) T cells (section 2.4).

Presentation of allergen under conditions that promote Th1-dominant immune responses offers an alternative to the induction of T cell tolerance for downregulating Th2 cytokine mediated immunity. In the final section the ability of different antigen delivery systems for HDM allergens and their effects on the qualitative nature of allergen immunity are investigated.

2.2 MODULATION OF ACCESSORY/CO-RECEPTOR MOLECULES

2.2.1 Introduction

The adhesion molecule LFA-1 and the coreceptor CD4 both enhance adhesion between and contribute to T cell activation. Antibodies against LFA-1 and CD4 can inhibit responses and, furthermore, the administration of non-depleting anti-CD4 antibodies in the presence of specific antigen leads to the induction of long-lasting T cell tolerance. There is some evidence that this phenomenon results in a "reprogramming" of the immune response mediated by the production of inhibitory and/or Th2 cytokines. The experiments reported here were designed to determine if the dependence of HDM specific Th2 cells on the costimulatory/coreceptor properties of LFA-1 and CD4 was different to that of Th0 and Th1 cells.

Faith A, Higgins JA, O'Hehir RE, Lamb JR: Differential dependence of Th0, Th1 and Th2 $CD4^+$ T cells on co-stimulatory activity provided by accessory molecule LFA-1. *Clin Exp Allergy* 1995, **25**:1163-1170.

Lamb JR, Faith A, Higgins JA, Verhoef A, Schneider P, Yssel H, O'Hehir RE: Clonal analysis of CD4 mediated accessory function on the effector activity of human CD4+ T cell subsets. *Clin Exp Allergy* 1995, **25**:839-847.

2.2.2 Summary

Human Th2 cells exhibited a greater requirement for the costimulatory activity of LFA-1 than either Th0 or Th1 cells. Similarly, functional modulation by anti-CD4 antibodies has different effects the various functional subsets of CD4+ T cells. Antigen induced IL-4 production was more resistant than IFN-γ to inhibition by anti-CD4 antibodies and certain antibodies were able to dissociate IFN-γ and IL-4 production and could inhibit IFN-γ without blocking antigen dependent proliferation. No evidence was obtained from these *in vitro* studies to suggest that anti-CD4 antibodies were able to induce T cell anergy.

2.2.3 Related publications

Faith A, O'Hehir RE, Malkovsky M, Lamb JR: Analysis of the basis of resistance and susceptibility of CD4+ T cells to HIV-gp120 induced anergy. *Immunology* 1992, **76:**1-8.

Faith A, O'Hehir RE, Yssel H, Lamb JR: Reversal of the inhibitory effects of HIV-gp120 on CD4+ T cells by stimulation through the CD28 pathway. *Clin Exp Immunol* 1996, **105**:225-230.

2.3 ANTIGEN MEDIATED MODULATION OF T CELL FUNCTION

2.3.1 INHIBITION OF T CELL PROLIFERATION

2.3.1.1 Introduction

The ability of certain peptides to bind with high affinity to selected class II MHC molecules has drawn attention to their potential to inhibit the presentation of "disease associated" epitopes. Competitor peptides can prevent experimental autoimmune encephalomyelitis (EAE) triggered by immunisation with myelin basic protein. Furthermore, in EAE it has been reported that T cells capable of mediating the disease have restricted TCR usage and that peptides derived from the CDR2 domain of the appropriate TCR-Vβ gene segment can prevent the induction and progression of disease. However, whether this treatment is effective in the human disease (multiple sclerosis) is not clear. Here the results of experiments are reported in which

both competitor peptides and TCR-Vβ derived peptides were investigated for their ability to inhibit the *in vitro* response of human CD4+ T cells from atopic and non-atopic individuals to HDM.

O'Hehir RE, Busch R, Rothbard JB, Lamb JR: An *in vitro* model of peptide-mediated immunomodulation of the human T cell response to *Dermatophagoides* spp. (house dust mite). *J Allergy Clin Immunol* 1991, **87**:1120-1127.

Hawrylowicz CM, Jarman ER, Guida L, O'Hehir RE, Lamb JR: T-cell receptor peptides that inhibit the T-cell response to allergen induce transforming growth factor-β_1 production. *J Allergy Clin Immunol* 1996, **97**:707-709.

2.3.1.2 Summary

In the first of the two papers presented here peptide competition at the level of MHC class II was investigated. An analogue of the influenza HA peptide (HA 307-319) with 309Y replaced by S was non-stimulatory in polyclonal T cell cultures and inhibited the proliferation of DRB1 and DRB3 restricted HDM specific T cell clones. Furthermore, the analogue downregulated polyclonal T cell responses induced by HDM but not other recall antigens.
From the analysis of TCR usage in HDM allergy a peptide based on the CDR2 of Vβ3 (see section 1.2.3.2) was synthesized and demonstrated to inhibit HDM induced polyclonal T cell responses. T cell responses to mycobacterial antigens were unaffected by the addition of the peptide.

While these approaches are of theoretical interest it is unlikely that they will have any practical application in allergen immunotherapy.

2.3.1.3 Related publications

Eckels DD, Gorski J, Rothbard JB, Lamb JR: Peptide mediated modulation of T cell allorecognition. *Proc Natl Acad Sci USA* 1988, **85**:8191-8195.

2.3.2. ALTERED T CELL RECEPTOR LIGANDS

2.3.2.1 Introduction

Generating peptide analogues with partial agonist activity in that they induce only selected T cell effector functions, for example cytokine production in the absence of proliferation, can be achieved by the substitution of conservative amino acids. The substitution of residues that make contact with either TCR

or MHC class II have been associated with the modulation of T cell effector function. Th0 cells are a marked component of the allergen specific T cell repertoire in allergic as well as normal individuals. Thus, the capacity to signal allergen specific Th0 cells to promote the production of IFN-γ and not Th2 cytokines may contribute to the downregulation of allergic inflammation. In the experiments reported here the ability of altered TCR ligands to modulate cytokine production by human Th0 cells was investigated in different antigen systems.

Tsitoura DC, Verhoef A, Gelder CM, O'Hehir RE, Lamb JR: Altered T cell ligands derived from a major house dust mite allergen enhance IFN-γ but not IL-4 production by human $CD4^+$ T cells. *J Immunol* 1996, **157**:2160-2165.

Tsitoura DC, Gelder CM, Kemeny DM, Lamb JR: Regulation of cytokine production by human Th0 cells following stimulation with peptide analogues: differential expression of TGF-β in activation and anergy. *Immunology* 1997, **92**:10-19.

2.3.2.2 Summary

In the first of the papers, analogues of an immunodominant epitope of Der p 2, residues 28-40 were synthesized with sequential alanine substitutions and their effect on proliferation and cytokine production evaluated for both Th0 and Th2 cells. Certain analogues could dissociate proliferation from IL-4 production by Th2 cells. There were examples in which analogues could inhibit proliferation and cytokine production by Th0 cells. The p 2, 28-40 peptide analogues with A at positions 34 and 36 altered the IFN-γ:IL-4 ratio of Th0 cells by selectively enhancing IFN-γ secretion.

In addition to promoting Th1 cytokine production there is evidence to suggest that altered TCR ligands may selectively induce the synthesis of inhibitory cytokines. The second paper in this section addressed this issue. It was observed for human Th0 cells reactive with HA306-318 that certain amino acid substitutions, which failed to induce proliferation, stimulated TGF-β production. At present no *in vivo* studies in experimental models have been carried out in order to establish if altered TCR ligands can modulate allergen induced Th2 immunity either systemically or locally in the respiratory tract.

2.3.2.3 Related publications

Lamb JR, Higgins JA, Hetzel C, Hayball JD, Lake RA, O'Hehir RE: The effects of changes at peptide residues contacting MHC class II or TcR on antigen recognition and human T_H0 cell effector function. *Immunology* 1995, **85**:447-455.

2.3.3 ANALYSIS OF *IN VITRO* ANTIGEN-INDUCED SPECIFIC ANERGY

2.3.3.1 Introduction

The clonal distribution of antigen specific receptors on lymphocytes offers the potential to tolerise selected population of cells. A principal aim of specific immunotherapy is to vaccinate with allergen or its derivatives under conditions that will inhibit the allergen specific response leaving the remainder of the immune system functionally intact. With this in mind an *in vitro* model using human CD4+ T cells was developed to investigate protocols for inducing antigen specific unresponsiveness. In this section of thesis, following the description of the model, characterisation of the cellular and molecular basis of T cell anergy is reported. The influence of ligand structure and the requirement for class II MHC was investigated for anergy mediated by conventional peptide antigens and bacterial superantigens. The effects of anergy on the expression of T cell surface proteins and the modulation of the functional phenotype, namely cytokine production are reported. The final three papers report on biochemical changes and transcriptional regulation in T cell anergy.

Lamb JR, Skidmore BJ, Green N, Chiller J, Feldmann M: Induction of tolerance in influenza virus-immune T lymphocyte clones with synthetic peptides of influenza haemagglutinin. *J Exp Med* 1983, **157**:1434-1447.

Tsitoura DC, Holter W, Cerwenka A, Gelder CM, Lamb JR: The induction of anergy in human Th0 cells by stimulation with altered T cell antigen receptor ligands. *J Immunol* 1996, **156**:2801-2808.

O'Hehir RE, Lamb JR: Induction of specific clonal anergy in human T lymphocytes by *Staphylococcus aureus* enterotoxins. *Proc Natl Acad Sci USA* 1990, **87**:8884-8888.

Lamb JR, Feldmann M: Essential requirement for major histocompatibility complex recognition of T-cell tolerance induction. *Nature* 1984, **308**:72-74.

Higgins JA, Lamb JR, Hayball JD, Marsh S, Tonks S, Rosen-Bronson S, Bodmer JA, O'Hehir RE: Peptide-induced non-responsiveness of HLA-DP restricted human T cells with *Dermatophagoides* spp. (house dust mite). *J Allergy Clin Immunol* 1992, **90**:749-756.

Hewitt CRA, Lamb JR, Hayball J, Hill M, Owen MJ, O'Hehir RE: MHC independent clonal T cell anergy by direct interaction of *Staphylococcus aureus* enterotoxin B with the T cell antigen receptor. *J Exp Med* 1992, **175**:1493-1501.

Zanders ED, Lamb JR, Feldmann M, Green N, Beverley PCL: Tolerance of T-cell clones is associated with membrane antigen changes. *Nature* 1983, **303**:625-627.

Lamb JR, Zanders ED, Sewell W, Crumpton MJ, Feldmann M, Owen MJ: Antigen-specific T cell unresponsiveness in cloned helper T cells mediated via the CD2 or CD3/Ti receptor pathways. *Eur J Immunol* 1987, **17**:1641-1644.

O'Hehir RE, Yssel H, Verma S, de Vries JE, Spits H, Lamb JR: Clonal analysis of differential lymphokine production in peptide and superantigen induced T cell anergy. *Int Immunol* 1991, **3**:819-826.

Schall TJ, O'Hehir RE, Goeddel DV, Lamb JR: Uncoupling of cytokine mRNA expression and protein secretion during the induction phase of T cell anergy. *J Immunol* 1992, **148**:381-387.

Essery G, Feldmann M, Lamb JR: Interleukin-2 can prevent and reverse antigen-induced unresponsiveness in cloned T lymphocytes. *Immunology* 1988, **64**:413-417.

Lake RA, O'Hehir RE, Verhoef A, Lamb JR: CD28 mRNA rapidly decays when activated T cells are functionally anergized with specific peptide. *Int Immunol* 1993, **5**:461-466.

Zanders ED, Feldmann M, Lamb JR: Biochemical events initiated by exposure of human T lymphocyte clones to immunogenic and tolerogenic concentrations of antigen. *Eur J Immunol* 1985, **15**:302-305.

Wotton D, Higgins JA, O'Hehir RE, Lamb JR, Lake RA: Differential induction of NF-AT complex during restimulation and induction of T-cell anergy. *Human Immunol* 1995, **42**:95-102.

2.3.2.2 Summary

Following exposure to supraimmunogenic concentrations of peptide in the presence and absence of APCs human cloned CD4+ T cells become unresponsive to antigenic restimulation and fail to prolferate or provide B cell help. The loss proliferation is not due to cell death since the T cells remain responsive to exogneous IL-2 after peptide treatment. The induction of anergy can be blocked with anti-MHC class II antibodies confirming the requirement for MHC class II dependent antigen recognition. This observation suggested that peptide may bind initially to class II MHC and then is presented to surrounding T cells of the same specificity and in the absence of the appropriate costimulatory signals induces unresponsiveness. In contrast, superantigen mediated anergy is independent of MHC binding and occurs through direct interaction with the TCR but mediates the same functional outcome.

The induction of anergy was associated with complex changes in the cell surface phenotype, most notably TCR and CD28 were downregulated, whereas CD25 and CTLA-4 were upregulated. No change in the expression of CD40L, CD80 or CD86 was observed. As regards changes in the functional phenotype these are also complex. Based on the measurement of cytokine specific transcripts in T cells undergoing activation, tolerance or that were in a resting state it appeared that hyperresponsiveness precedes the induction of anergy. However, release of the soluble protein did not always parallel mRNA levels and this was well illustrated from the analysis of IL-8 production. These experiments were extended and the production of cytokines was analysed when T cells pretreated with anergising concentrations were rechallenged with antigen and APCs. In this situation the synthesis of the majority of the cytokines analysed was inhibited with the exception of IFN-γ, TGF-β and IL-10. Although the immunosuppressive

activity of TGF-β and IL-10 could account for the functional inhibition observed, however, it appears that their production alone is not sufficient and cell contact was also required.

CD28 transduces an efficient costimulatory signal for the activation of T cells and in the absence of which ligation of TCR by peptide/MHC class II complexes results in anergy. In addition, CD28 expression is differentially regulated during activation and the induction of anergy. The induction of anergy in resting and fully activated CD4+ T cells was accompanied by a profound downregulation of mRNA and surface expression of CD28. The ligation of CD28 affects IL-2 synthesis by the generation of a nuclear factor complex, CD28RC, that increases transcription from the IL-2 gene promoter. The modulation of this and other selected transcription factors that regulate expression of the TCR-β chain and IL-2 genes during the induction of anergy and restimulation of anergic T cells was investigated. The results of these experiments revealed that NF-AT was markedly lower in anergic than activated cells, however, this may be a consequence of reduced calcium signalling.

2.3.2.3 Related publications

Zanders ED, Feldmann M, Green N, Lamb JR: Direct evaluation of antigen binding to human T lymphocyte clones: involvement of major histocompatibility complex products in antigen binding. *Eur J Immunol* 1984, **14**:1101-1105.

Lamb JR, Feldmann M, Green N, Lerner RA: Influence of antigen structure on the activation and induction of unresponsiveness in cloned human T lymphocytes. *Immunology* 1986, **57**:331-335.

Bal A, McIndoe A, Denton G, Hudson D, Lombardi G, Lamb JR, Lechler R: Antigen presentation by keratinocytes induces tolerance in human T cells. *Eur J Immunol* 1990, **20:**1893-1899.

O'Hehir RE, Aguilar BA, Schmidt TJ, Gollnick SO, Lamb JR: Functional inactivation of *Dermatophagoides* spp. (house dust mite) reactive T cell clones. *Clin Exp Allergy* 1991, **21**: 209-215.

Yssel H, Fasler S, Lamb JR, de Vries JE: Induction of non-responsiveness in human allergen specific Th2 cells. *Curr Opin Immunol* 1994, **6**:847-852.

O'Hehir RE, Lake RA, Schall TJ, Yssel H, Panagiotopolou E, Lamb JR: Regulation of cytokine and chemokine transcription in human Th2 cells during the induction phase of anergy. *Clin Exp Allergy* 1996, **26**:20-28.

Holan V, Lamb JR, Malkovsky M: Immunological tolerance and lymphokines. *CRC Crit Rev Immunol* 1991, **10**:481-493.

2.3.4 INDUCTION OF PERIPHERAL TOLERANCE *IN VIVO*

2.3.4.1 Introduction

Although *in vitro* models are useful in the analysis of T cell unresponsiveness they have obvious limitations and, therefore, the tolerogenic activity of peptides was investigated *in vivo* in a murine model of specific immunity to HDM.

Analysis of responses to Der p 1 revealed that H-2^b mice were high responders with the immunodominant T cell epitope contained within residues 110-131 (p 1, 110-131). Weaker responses were observed to the sequences 15-29, 81-102 and 197-212, which were termed minor determinants. The ability of peptides to induce peripheral T cell tolerance following intranasal administration was investigated and papers that describe the cellular characterisation and address the molecular mechanisms of tolerance are included. The final paper in the section reports a clinical study in which the effect of grass pollen immunotherapy on proliferative responses and IL-5 cytokine production was examined to examine whether or not tolerance and/or immune reprogramming contributed to successful immunotherapy.

Hoyne G, O'Hehir RE, Wraith DG, Thomas WR, Lamb JR: Inhibition of T cell and antibody responses to house dust mite allergen by inhalation of the dominant T cell epitope in naive and sensitized mice. *J Exp Med* 1993, **178**:1783-1788.

Hoyne GF, Askonas BA, Hetzel C, Thomas WR, Lamb JR: Regulation of house dust mite responses by intranasally administered peptide: transient activation of $CD4^+$ T cells precedes the development of tolerance *in vivo*. *Int Immunol* 1996, **8**:335-342.

Hoyne GF, Jarnicki AG, Thomas WR, Lamb JR: Characterisation of the specificity and duration of T cell tolerance to intranasally administered peptides in mice: a role for intramolecular epitope suppression. *Int Immunol* 1997, **9**:1165-1173.

Till S, Walker S, Dickason R, Huston D, O' Brien F, Lamb J, Kay AB, Corrigan C, Durham S: Interleukin-5 production by allergen-stimulated T cells following grass pollen immunotherapy for seasonal allergic rhinitis. *Clin Exp Immunol* 1997, **110**:114-121.

2.3.4.2 Summary

The intranasal administration of the immunodominant peptide p 1, 110-131 in $H\text{-}2^b$ mice induced profound antigen specific peripheral T cell tolerance that was long-lasting. There was a reduction in all T cell derived cytokines and modulation of delayed type hypersensitivity in the tolerant mice. Tolerance to p 1, 110-131 inhibited the response of T cells reactive with all epitopes on Der p 1 provided that tolerant mice were immunised with the intact protein. This phenomenon is termed linked suppression has been described for other models of peripheral tolerance to transplantation and self-antigens. The nature of the signals that mediate linked suppression and are poorly understood and it has been proposed that it arises from antigen dependent production of inhibitory cytokines derived from regulatory T cells that suppress the response of naive T cells. However, T cells isolated from tolerant mice failed to produce immunosuppressive cytokines when restimulated with antigen *in vitro* suggesting that linked suppression is mediated through an alternative mechanism, perhaps involving direct cell contact (see section 2.4). Serological inhibition *in vitro*, the induction of tolerance in CD8 gene deficient mice together with the ability of CD4+ T cells to transfer the effect to naive recipients all imply that regulatory cells were induced and that the phenomenon of linked suppression was mediated by a subset of CD4+ T cells.

There are two additional aspects of this model of peptide mediated tolerance induced via the mucosa of the airways that warrant comment in the context of clinical immunotherapy. Firstly, transient T cell activation occurs before tolerance develops. Secondly, T cells from tolerant mice were unable to support HDM specific antibody production *in vitro* cultures of T cells, however, *in vivo* IgE responses were unaffected. Several clinical trials of allergen immunotherapy have demonstrated that specific IgE is elevated at the start of therapy but may remain at relatively high levels even though the patients report an improvement in symptoms.

In the clinical study reported here it was observed that grass pollen immunotherapy in patients who had received 6 to 7 years of continuous conventional immunotherapy or had received 3 to 4 years of conventional immunotherapy followed by 3 years of placebo treatment abogated the cutaneous late phase responses. However, this was not accompanied by a reduction in IL-5 production or proliferation by peripheral blood mononuclear cells (PBMCs), neither was IFN-γ synthesis enhanced. Thus, clinical efficacy of immunotherapy may require only modulation of the local Th2 immunity.

2.3.3.3 Related publications

Hoyne GF, Kristensen NM, Yssel H, Lamb JR: Peptide modulation of allergen specific immune responses. *Curr Opin Immunol* 1995, **7**:757-761.

2.3.4 INDUCTION OF REGULATORY (INHIBITORY) CELLS

2.3.4.1 Introduction

Understanding the mechanisms of peripheral tolerance to inhaled antigens has attracted attention in respect to defining and regulating chronic respiratory inflammation. It now appears that regulatory (inhibitory) cells may contribute to the induction immunological tolerance in the airways. The phenotype of these cells is controversial. In some reports the inhibition is mediated by CD8+ T cells, expressing either $\alpha\beta$ or $\gamma\delta$ TCR, whereas in others the regulatory cells are a subset of CD4+ cells.

Here two sets of studies are described. In the first with human PBMCs as responder cells and autologous cloned T cells as stimulator cells regulatory cells were isolated and characterised. In addition, human PBMCs from atopic and non-atopic individuals were stimulated with TCR-Vβ CDR2 derived peptides (see section 2.3.1). The second study focuses on the *in*

vivo selection of regulatory CD4+ T cells induced in mice rendered tolerant to Der p 1 by intranasal delivery of peptides (see section 2.3.3).

Lamb JR, Feldmann M: A human suppressor T cell clones which recognizes an autologous helper T cell clone. *Nature* 1982, **300**:456-458.

Jarman ER, Hawrylowicz CM, Panagiotopolou E, O'Hehir ER, Lamb JR: Inhibition of human T-cell responses to house dust mite allergens by a T-cell receptor peptide. *J Allergy Clin Immunol* 1994, **94**:844-852.

Hoyne GF, Dallman MJ, Lamb JR: Linked suppression in peripheral T cell tolerance to the house dust mite derived allergen Der p 1. *Int Arch Allergy Immunol* 1999, **118**:122-124.

2.3.4.2 Summary

In order to establish if regulatory T cells can be detected in peripheral T cell repertoire in man PBMCs were cultured with autologous T cells. In this way it was possible to generate T cells that could inhibit the response of autologous CD4+ helper T cells. In addition, as described above (section 2.3.1.1), a TCR-Vβ3 CDR2 derived peptide can inhibit HDM induced proliferation of polyclonal T cells from atopic individuals. Analysis revealed that this effect was mediated by CD8+ T cells that produced TGF-β, but not the other immunosuppressive cytokines IL-4 or IL-10.

Intranasal administration of the immunodominant peptide (p 1, 110-131) resulted in the induction of tolerance that can be transferred to naive recipients by CD4+ T cells and, furthermore, these cells mediated linked suppression. It has been proposed in other systems of peripheral tolerance in which linked suppression is also observed that the secretion of inhibitory cytokines may provide the negative signals that mediate this effect. However, the failure to detect the production of these cytokines in mice tolerised by intranasal peptide suggested alternative mechanisms may operate which are dependent on direct cell contact between regulatory and naive T cells. Cognate interactions involving the Notch signalling pathway were demonstrated to mediate linked suppression.

2.3.4.3 Related publications

Lamb JR, Eckels DD, Ketterer EA, Sell TW, Woody JN: Antigen specific human T lymphocyte clones: mechanisms of inhibition of proliferative responses by xenoantiserum to human non-polymorphic HLA-DR antigens. *J Immunol* 1982, **129**:1085-1090.

Naor D, Essery G, Tarcic N, Kahan M, Lamb JR, Feldmann M: Specific interactions between a human CD4+ clone and autologous bifunctional immunoregulatory clones. *Immunol Rev* 1990, **116**: 63-85.

2.3.5 SELECTIVE ANTIGEN PRESENTATION BY VACCINE VECTORS

2.3.5.1 Introduction

An alternative approach to the induction of tolerance presentation of allergen under conditions that promote Th1 cytokines may facilitate the downregulation of allergic inflammation. For these experiments two distinct vector systems were selected because they have already been used in man. Firstly, particulate antigen presentation based on the Ty p1 protein, which forms virus-like particles (VLPs) when expressed in yeast was analysed. VLPs are efficient in generating both specific CD4+ Th1-type and CD8+ cytotoxic T cell (CTL) responses.

The second vector analysed is mycobacteria. They are known to be highly immunostimulatory and through their uptake and presentation by macrophages favour the stimulation of Th1-type responses by the production of IL-12 and IFN-γ. Therefore, mycobacteria were an appropriate live vaccine vector to investigate for the expression and presentation of HDM genes with which to induce Th1 cells.

Harris SJ, Roth J-F, Savage N, Woodrow SA, Hoyne GF, Lamb JR, Layton GT: Prediction of murine MHC class I epitopes in a major house dust mite allergen and induction of T1-type $CD8^+$ T cell responses. *Int Immunol* 1997, **9**:273-280.

Lamb JR: Peptide-mediated regulation of allergen-specific immune responses. *Res Immunol* 1998, **149**:235-240.

Hetzel C, Janssen R, Ely SJ, Kristensen NM, Bunting K, Cooper JB, Lamb JR, Young DB, Thole J: An epitope delivery system for use with recombinant mycobacteria. *Infect Immun* 1998, **66**:3643-3648.

2.3.5.2 Summary

Immunisation of H-2^b mice with VLPs expressing p 1, 111-139 induced Th1-type CD4+ T cells that responded *in vitro* to both the specific peptide and the native Der p 1 protein. Furthermore, p 1, 111-139-VLPs stimulated a 111-119 specific H-$2D^b$ restricted CD8+ CTL response that was long-lasting. Both Der p 1 specific CTL and T helper cells produced high levels of IFN-γ. The role of specific CD8+ T cells in the regulation of allergic responses in man is unclear, although there are reports of that the number of CD8+ T cells in the periphery increases following desensitisation.

Antigenic determinants from HDM proteins were engineered into an extended loop of the superoxide dismutase molecule of *M. tuberculosis*, and expressed in *M. vaccae*, a non-pathogenic species. H-2^b mice that were primed with recombinant *M. vaccae* expressing p 1,111-139 (r*M. vaccae* -Der p 1) generated specific immunity predominantly of the Th1 phenotype, with little or no IL-5 detectable. These experiments were extended and mice injected with r*M. vaccae* -Der p 1 were subsequently immunised with peptide to induce a specific Th2-dominant response. Enhanced levels of IFN-γ together with a reduction in IL-5 synthesis were observed in these mice compared to the controls. This indicated that the pretreatment with r*M. vaccae* -Der p 1 had primed mice for Th1 immunity that was reactivated when the mice were rechallenged with antigen under conditions selected to induce Th2 responses.

2.3.5.3 Related publications

Abou-Zeid C, Gares M-P, Inwald J, Janssen, R, Zhang Y, Young DB, Hetzel C, Lamb JR, Baldwin SL, Orme IM, Yeremeev V, Nikonenko, BV, Apt AS: Induction of a type 1 immune response to a recombinant antigen from *Mycobacterium tuberculosis* expressed in *Mycobacterium vaccae*. *Infect Immun* 1997, **65**:1856-1862.

APPENDIX

The papers included in the appendix are reproduced with permission from the following journals:

Cell
Clinical Experimental Allergy
Clinical Experimental Immunology
EMBO Journal
European Journal of Immunology
Human Immunology
Immunogenetics
Immunology
Immunology Today
Infection and Immunology
International Archives of Allergy and Immunology
International Immunology
Journal of Allergy Clinical Immunology
Journal of Experimental Medicine
Journal of Immunology
Journal of Virology
Nature
Proceedings of the National Academy of Sciences of the United States of America
Research in Immunology

1.2 ANALYSIS OF THE ANTIGEN SPECIFICITY OF T CELL RESPONSES

1.2.1 INFLUENZA VIRAL ANTIGENS

Lamb JR et al: *J Immunol* 1982, **128**:233-238.
Lamb JR et al: *J Immunol* 1982, **128**:1428-1432.
Lamb JR et al: *Nature* 1982, **300**:66-69.
Gelder CM et al: *J Virol* 1995, **69**:7497-7506.
Gelder CM et al: *J Virol* 1996, **70**:4787-4790.

0022-1767/82/1281-0233$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 128, No. 1, January 1982
Printed in U.S.A.

ANTIGEN-SPECIFIC HUMAN T LYMPHOCYTE CLONES: INDUCTION, ANTIGEN SPECIFICITY, AND MHC RESTRICTION OF INFLUENZA VIRUS-IMMUNE CLONES[1]

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Human peripheral blood lymphocytes from an *HLA-Dw1,3* individual were primed *in vitro* with influenza A virus (A/Texas/1-77/x-49) and subsequently cloned by limiting dilution in TCGF. Of the 96 TLCs originally obtained, nine were characterized in detail. TLCs were antigen specific, responding to influenza A virus, not to influenza B, TGAL, GAT, tetanus toxoid, or KLH, and only when antigen was presented by cells unable to form rosettes with AET-treated SRBC. Presentation of antigen by unseparated PBL often resulted in significant "back stimulation," probably via production of growth factors. The MHC requirements for the induction of TLC proliferation were analyzed. Of four representative clones analyzed, three required *Dw1*;*DR1* compatibility for successful presentation of viral antigens by a panel of antigen-presenting cells. In contrast, one TLC showed an unusual pattern of response that could not be correlated to a particular *HLA* haplotype. Monoclonal anti-T cell antibody analysis of the surface phenotype of two TLCs maintained in continuous culture for 5 mo indicated that they were OKT3$^+$, 4$^-$, and 8$^-$, consistent with an inducer/helper phenotype. To confirm the clonal nature of TLCs, data on the functional properties of TLC subclones are also presented.

The analysis at the molecular level of T cell interactions in the regulation of immune function requires expanded populations of monoclonal T lymphocytes. A number of approaches have been utilized to obtain enriched populations; for example, antisera reactive with cell-surface determinants have defined the characteristics of some T lymphocyte subsets both in mouse (1, 2) and in man (3–5). By using such antisera, and by either positive or negative selection, certain T cell types can be enriched (6–8), although these techniques fail to isolate monoclonal populations. In another approach, somatic cell hybridizations between normal murine T cells and thymomas resulting in T cell hybrids with specific suppressor (9) or helper (10) activity have been reported. However, such techniques are not sufficiently developed to allow the monoclonal expansion of all T cell subsets and are currently not available for analyzing human T lymphocyte function. Recently, the recognition that supernatants from mitogen-stimulated lymphocyte cultures, which contain T cell growth factor (TCGF),[5] can maintain the growth of T lymphocytes *in vitro* (11, 12) has allowed the expansion and maintenance of functional antigen-specific T cell subpopulations in long-term culture (13–15). Consequently, in the mouse it has been possible to prepare antigen-reactive clonse of T cells that recognize specific alloantigenic determinants (16), particulate antigens such as sheep erythrocytes (SRBC) (17), or soluble protein antigens (18).

In the human, the majority of early studies utilized proliferation of peripheral blood mononuclear cell (PBL) populations to antigens such as purified protein derivative (PPD) to analyze cell interactions (19, 20). More recently, long-term cultures of human T lymphocytes that are dependent on TCGF for continued growth and that are antigen specific have been reported (21, 22). Furthermore, it has also been demonstrated that human T cell lines specific for soluble antigens (23, 24) or alloantigens (25) can be cloned. In human studies evidence has accumulated that *HLA-D*;*DR* sharing is required between T lymphocytes and antigen-presenting cells (APC) in order for antigen-specific proliferation to occur (20, 22, 26, 27). As shown recently, the use of clones of human T lymphocytes eliminates the problem of alloreactivity (24), making possible the analysis of genetic requirements for the induction of T cell response using allogeneic panels of cell donors. In the studies reported here, we describe the system for the generation and characterization of T lymphocyte clones (TLC) that have antigen specificity for influenza viral proteins. In addition, we present evidence that the majority of TLC and subclones studied require *HLA-D/DR*-identical APC in addition to virus in order for triggering to occur. However, for one antigen-specific TLC an unusual pattern of genetic restriction was observed.

MATERIALS AND METHODS

Cells. PBL were obtained from whole blood diluted with an equal volume of RPMI 1640 (Grand Island Biologicals Company, Grand Island, NY) and

Received for publication September 17, 1981.
Accepted for publication October 14, 1981.
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[1] This work was supported by the Office of Naval Research Contracts N000-14-77C0748, 77C0747, 80K0909, and 79C0886 and Naval Medical Research and Development Command Work Unit M0095PN001, 1028, and 0045. The opinions expressed herein are those of the authors and are not to be construed as those of the Department of the Navy or the Department of Defense.
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[5] Abbreviations used in this paper: AET-SRBC, S-2-aminoethylisothiouronium bromide-treated SRBC; APC, antigen-presenting cell; DNV, double normalized value; E, erythrocyte; E$^+$, mononuclear cells that form rosettes with AET-SRBC; E$^-$, non-AET-SRBC rosette-forming cells; GAT, L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10}; ^{3}HTdR, tritiated methylthymidine; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid; HLA, human MHC; HTC, homozygous typing cell; KLH, keyhole limpet hemocyanin; MHC, major histocompatibility complex; PBL, peripheral blood mononuclear cells; PWM, pokeweed mitogen; TCGF, T cell growth factor; TGAL, (L-tyrosine:L-glutamic acid) poly DL-alanine:poly-L-lysine; TLC, T lymphocyte clone; T. Tox., tetanus toxoid; HAU, hemagglutinating units; FITC, fluorescein isothiocyanate.

centrifuged over Ficoll-Hypaque (Sigma Chemical Company, St. Louis, MO, and Winthrop Laboratories, New York, NY) at 400 × G for 30 min. After washing, the cells were resuspended to 10^7/ml in RPMI 1640 medium supplemented with 10% pooled human A$^+$ serum, 2 mM L-glutamine, 25 mM HEPES* buffer, 50 μg/ml gentamicin, 25 IU/ml sodium heparin, 1 mM sodium pyruvate, and 7.5% v/v dimethylsulfoxide at 4°C. The cells were frozen at −1°C/min for 20 min using a rate-controlled freezer (Cryson, Associated Biomedic Systems, Buffalo, NY), then at −50°C/min down to −80°C. After this procedure, the cells were transferred to the vapor phase of a liquid nitrogen freezer (MVE Cryogenics, New Prague, MN) and stored at −180°C until required.

Antigens. Attenuated influenza A (A/Texas/1-77/x-49) and B (B/Singapore/222/79) strain viruses were kindly prepared by M. Phelan and W. E. Barthlow, Division of Virology, Bureau of Biologics, NIH, Bethesda, MD. The polymers L-glutamic acid^{60}:L-alanine^{30}:L-tyrosine^{10} (GAT) and (L-tyrosine:L-glutamic acid) poly DL-alanine:poly-L-lysine (TGAL; Lot MCB) were purchased from Miles Laboratories, Inc., Miles Research Division, Elkhart, IN. Keyhole limpet hemocyanin (KLH) was the generous gift of Dr. M. Rittenberg, Portland, OR. Tetanus toxoid (T. Tox.) was purchased from Massachusetts Biological Laboratories, Boston, MA.

Antigen activation of PBL. PBL at 5 × 10^6 cells/ml in RPMI 1640 (GIBCO) containing 10% screened, pooled human A$^+$ serum, 2 mM L-glutamine, 25 mM HEPES buffer, 50 μg/ml gentamicin, 25 IU/ml Na-heparin, and 1 mM Na-pyruvate were stimulated with influenza A virus at 5 hemagglutinating units (HAU)/ml. The concentration of virus required to produce optimal stimulation of PBL, assayed by incorporation of tritiated methylthymidine (^{3}HTdR), varied for different virus preparations, but was always in the range of 2 to 5 HAU/ml. After mixing, 0.2-ml aliquots of the cell suspensions were plated into 96-well, U-bottom tissue culture trays (Linbro Scientific Company, Hamden, CT) and incubated for 6 days at 37°C in a 5% CO_2/air mixture.

T cell growth factor (TCGF). PBL from screened donors were cultured at 1 × 10^6/ml in RPMI 1640 supplemented with 0.1% purified phytohemagglutinin-P (PHA-P; Difco Laboratories, Detroit, MI) and 1% autologous plasma (28). After 48 hr, supernatants were harvested, passed through 0.22-μm filters, and assayed for their ability to support the growth of a TCGF-dependent cell line assayed by ^{3}HTdR incorporation. Acceptable lots of TCGF were stored at 4°C or diluted as required to 20% v/v in 10% AB plasma and supplemented RPMI 1640.

Cloning of influenza-specific lymphocytes. After 6 days culture in the presence of influenza virus, cells were harvested, resuspended over 35 to 40% Percoll (Pharmacia, Uppsala, Sweden) in 12 × 75 mm sterile test tubes (Falcon Division, Becton Dickinson and Company, Cockeysville, MD), and centrifuged for 20 min at 200 × G. Cells at the interface were enriched two- to five-fold for lymphoblasts and, comprised 50 to 70% of the cells counted. Blast-enriched suspensions were diluted to 33⅓ cells/ml of medium containing 20% TCGF and plated in 10-μl aliquots in sterile 60-well Microtest II trays (Falcon). Ten thousand autologous cells were gamma irradiated (2500 rad, ^{137}Cs), combined with optimal concentrations of influenza virus, and added to wells containing blasts in 10-μl aliquots. Cultures were incubated for 7 days in humidified chambers at 37°C in 5% CO_2/air, after which growing wells were transferred to fresh medium (0.2 ml) containing 20% TCGF, irradiated autologous feeders (5 × 10^5/ml), and influenza virus in 96-well flat-bottom trays. After seven additional days of culture, the clones were transferred to 24-well trays (Linbro Scientific Company, Hamden, CT) containing the appropriate concentrations of TCGF, autologous feeders, and influenza in a total volume of 2 ml. Cultures received fresh TCGF every 3 to 4 days, alternating with pooled irradiated feeders (5 × 10^5/ml) without virus, and were thus maintained throughout the course of the experiments. Clones were allowed to grow 6 to 8 days after addition of feeder cells before testing in proliferative assays. For the preliminary screening of influenza-responsive clones, individual clones from 24-well trays were diluted 1:200, and the proliferative response in the presence of autologous gamma-irradiated PBL and influenza A virus was determined by the incorporation of ^{3}HTdR.

Proliferation assays. Five thousand TLC cells in 10% A$^+$ serum and supplemented medium were added to 96-well, U-bottom trays in 0.1-ml aliquots. Fractionated or unfractionated autologous PBL, as a source of APC, were suspended in medium containing 10% A$^+$ serum and optimum concentrations of antigen, and were dispensed in 0.1-ml aliquots to wells containing TLC cells. Cultures were incubated for 12 to 144 hr, pulsed for 8 to 18 hr with 1.0 μCi of ^{3}HTdR (New England Nuclear, Boston, MA), and harvested onto glass-fiber filters. Proliferation, as correlated with ^{3}HTdR incorporation, was measured by liquid scintillation spectroscopy. The results are expressed as the mean counts per minute (cpm) ± standard error of the mean (SEM) for triplicate cultures.

Fractionation of lymphocyte populations by SRBC rosetting. Erythrocyte (E) rosettes were formed with SRBC treated with S-2-aminoethylisothiouronium bromide hydrobromide (AET; Calbiochem, San Diego, CA) as previously described (29). One volume of packed SRBC was incubated with 5 vol of AET (40.2 mg/ml in distilled H_2O, pH 9.0) for 20 min at 37°C. One volume of PBL at 10^7 cells/ml was mixed with 2 vol of 2% AET-SRBC and 0.5 volumes of fetal calf serum (FCS). The suspension was centrifuged at 250 × G for 10 min and placed on ice for 60 min. After resuspension of the pellets by gentle rotation, the separation of E rosette-forming (E$^+$) from nonrosetting (E$^-$) lymphocytes was achieved by centrifugation (1500 × G, 15 min) over Percoll (ρ = 1.080 g/ml; Pharmacia). The E$^+$ cells were recovered from the interface and the E$^-$ cells from the pellet by lysis of SRBC with Gey's hemolytic solution (30). The E$^-$ cell population contained less than 1% E$^+$ cells. In proliferation assays, cultures were reconstituted with 5 × 10^4 E$^-$, 20 × 10^4 E$^-$, or 25 × 10^4 E$^-$ and E$^+$ cells. Comparable cultures were reconstituted with 25 × 10^4 irradiated PBL as a source of APC.

HLA typing. APC from panel members were serotyped according to HLA workshop specificities approved by the W. H. O. Nomenclature Committee for HLA workshop specificities as well as for MB and MT using more than 400 sera. The typing techniques used are described in detail elsewhere (31). The families and many panel members have been characterized in at least one International Histocompatibility Workshop. *HLA-D* typing was performed utilizing homozygous typing cells (HTC) from the 7th and 8th Workshop as well as local HTC. Cells were assigned a specificity (*Dw1-10*) if they were typed with two defined HTC with an average DNV of less than 40 (32).

Phenotypic analysis of TLC. Cloned T cells were pelleted by centrifugation at 300 to G for 10 min at 4°C and then resuspended to 10^7 cells/ml in medium supplemented with 10% FCS and 1 mg/ml Na azide at 4°C. To 100-μl aliquots of cells, 5 μl of monoclonal antibody were added, and the mixture was incubated for 30 min with agitation every 10 min. After two washes in cold medium, 10 μl of counterstaining reagent (either fluorescein isothiocyanate- [FITC; F(ab')$_2$-sheep anti-mouse-IgG, Cappel Laboratories, Cochranville, PA, or FITC-conjugated avidin) were added, incubated for 30 min on ice with agitation, and washed twice. Cells were resuspended in 2 ml of medium and analyzed for fluorescence on an Ortho Cytofluorograph, model 50H (Ortho Diagnostics, Boston, MA). The murine monoclonal antibodies and reagents used in these characterizations were as follows: OKT3,4,6,8 (Ortho); biotin-conjugated Leu 2A,3A, OR, FITC-avidin, and FITC-Leu 1 (Becton Dickinson, Sunnyvale, CA); HLA-framework (Bethesda Research Laboratories, Bethesda, MD); FITC-conjugated, pooled goat anti-human-Ig (F(ab')$_2$ (Kallestad Laboratories, Inc., Chaska, MN).

Subcloning. Subclones were induced using cloning procedures described above. T cells were plated at 0.3 cells/well in Terasaki plates with autologous irradiated PBL and influenza A virus in supplemented RPMI 1640 containing 20% TCGF. Cultures showing positive growth at 7 days were transferred to 96-well trays and finally to tissue culture flasks as described above.

Statistical methods. In order to objectively classify negative and positive responses, we have employed the Kurtosis test for outliers as suggested by Sheehy (33). This analysis was only applied to the major histocompatibility complex (MHC) restriction experiments.

RESULTS

Expansion and preliminary screening for influenza responsive clones. After initial stimulation with virus, lymphoblasts were plated in Terasaki plates and 12% of the wells contained growing cells after 7 days. Because the blast-enriched cells were plated at one cell every third well, approximately 35% of the total number of seeded cells proliferated. Furthermore, upon subsequent transfer to 96-well trays, 100% of the clones were still growing after seven additional days in culture.

A total of 96 individual TLC were cultured in the absence of TCGF with autologous irradiated PBL as a source of APC, and their responsiveness to influenza A virus was determined by ^{3}HTdR incorporation. In order to concentrate our efforts, only TLC that showed proliferation in excess of 10,000 cpm were investigated further. Indeed, 15 TLC were distributed within this category, whereas the remainder incorporated less than 10,000 cpm in the presence of influenza virus. To indicate that the lack of responsiveness of negative clones was not due to cell death, TLC were also cultured with TCGF. The proliferative response of a selected group of clones is shown in Table I. By fulfillment of the requirements outlined above, TLC 18, 50, 76, and 77 were considered negative, whereas the remainder (TLC 6, 24, 37, 53, 71, 69, 72, and 88) were considered positive.

The clones selected from the preliminary screening were cultured with APC and influenza A virus, and the proliferative

kinetics was determined by ^{3}HTdR incorporation at 12 to 144 hr after initiation of the culture (data not shown). Of the positive clones selected, all reached maximum ^{3}HTdR incorporation between 72 and 96 hr, and by 144 hr proliferation was not significantly above background. The magnitude of the specific response at 72 hr was fivefold greater than the nonspecific response to TCGF. TLC 50, however, responded much more vigorously to TCGF than specific antigen, suggesting that the clone was truly negative and that its failure to respond did not reflect cell death. The response of TLC to medium alone remained below 100 cpm.

Antigen specificity of T lymphocyte clones. The antigenic specificity of individual TLC was assayed by culturing clones with irradiated autologous PBL and various antigens (Table II). Although all positive clones (TLC 6, 24, 26, 37, 53, and 71) proliferated vigorously to influenza A virus, each TLC was also restimulated by at least one apparently unrelated antigen. Such ancillary reactions were generally less than 10% of the response to influenza A; however, they were significantly above background controls. When antigen was presented by irradiated AET-SRBC E$^-$ cells, TLC responded to specific antigen with virtually no cross-reactivity to unrelated antigens (Table III-*A*). The presence of peripheral blood T cells in the presenting-cell population, however, induced nonspecific proliferation of the TLC. Representative results of the nonspecific stimulation of TLC by contaminating T cells in the presenting-cell population are shown for TLC 53 (Table III-*B*). Without exception, TLC were not reactive to unrelated antigens when presented in the absence of T cells.

Presentation of antigen by HLA-D region-related and -unrelated cell types. A panel of E$^-$ APC were tested for their capacity to present influenza virus to T lymphocyte responder clones (Table IV). TLC 6, 37, and 53 were effectively restimulated only in the presence of virus and APC bearing *HLA-Dw1*; *DR1*. Proliferation induced by APC that did not express *Dw1*; *DR1* was less than 100 cpm and was not significantly different from negative controls of TLC cultured with antigen alone or medium. One other clone did not appear to be restricted by a known *D*;*DR*-type (Table IV). TLC 71 responded to influenza virus presented by APC from LNAT (autologous), DSMO, DBUD, and MLAU. No consistently common HLA antigen was found on these four cells. This pattern of presentation to TLC 71 was reproducible. TLC 50 was included as a negative, nonspecific control and was never induced to proliferate by

TABLE I

Distribution of influenza TLC into high, intermediate and low responders[a]

Clone No.	Proliferative Response of TLC				
	+ PBL (IR) + Flu	+ Flu	+ PBL (IR)	+ TCGF	Medium
	cpm ± SEM				
50	222 (58)[b]	10 (2)	24 (3)	8,492 (1,126)	39 (14)
76	219 (38)	11 (1)	15 (4)	4,870 (218)	15 (2)
18	1,280 (242)	13 (1)	34 (3)	4,427 (689)	17 (1)
77	2,105 (416)	14 (1)	18 (5)	5,261 (754)	13 (3)
69	4,136 (1,088)	14 (1)	112 (26)	3,578 (747)	33 (5)
88	4,774 (385)	9 (3)	26 (7)	3,753 (847)	13 (1)
6	36,149 (2,394)	23 (5)	134 (44)	10,845 (152)	17 (5)
24	12,005 (1,883)	18 (2)	118 (43)	2,078 (32)	20 (5)
26	14,243 (3,414)	15 (2)	32 (6)	3,637 (954)	17 (5)
37	24,534 (5,897)	16 (4)	639 (163)	7,142 (404)	5 (1)
53	27,051 (4,555)	13 (1)	1,549 (335)	7,870 (1,240)	17 (2)
71	32,689 (7,629)	11 (4)	2,248 (392)	7,703 (490)	37 (3)
72	15,567 (3,059)	18 (1)	42 (11)	1,375 (202)	25 (1)

Controls:	PBL (IR)			
	+ Flu	+ PWM	+ PHA	Alone
	11 (3)	16 (3)	16 (3)	8 (2)

[a] Unfractionated PBL from a high responder were stimulated with influenza A virus and cloned by limiting dilution. 96 TLCs were selected and expanded in liquid culture. A 1:200 dilution of 13 TLCs were stimulated with 5HA units/ml of influenza A in a 72-hr ^{3}HTdR incorporation assay in the presence of 25 × 10^3 irradiated (2500 rads) autologous PBL. Controls of irradiated autologous PBL cultured with influenza A, PWM, and PHA are shown.

[b] Values expressed as mean cpm (± SEM) of triplicate cultures.

TABLE II

Response of influenza A virus induced TLC to specific and unrelated antigens[a]

Antigen	Proliferative Response of TLC Clone No.							
	6	24	26	37	53	71	72	50
Influenza A (5 HA/ml)	*13,707*[b]	*9,549*	*11,935*	*11,049*	*8,130*	*9,475*	*3,191*	67
Influenza B (5 HA/ml)	120	33	45	116	59	101	32	55
GAT (500 μg/ml)	19	25	25	67	22	68	40	65
TGAL (1 mg/ml)	26	18	18	*147*	33	58	122	298
T. Toxoid (0.1 LF/ml)	*424*	*200*	*730*	*985*	*610*	*1,593*	37	64
KLH (80 μg/ml)	61	26	21	68	47	196	42	23
Medium	21	10	15	14	19	36	12	18
TCGF	2,197	689	1,266	1,143	1,213	2,011	363	1,476

[a] 5 × 10^3 T cells from 8 TLC were stimulated with influenza A (5 HA units/ml), influenza B (5 HA units/ml), GAT (500 μg/ml), TGAL (1 mg/ml), T. Tox (0.1 LF/ml) or KLH (80 μg/ml) together with 25 × 10^3 autologous irradiated PBL. Stimulation was assessed by the incorporation of ^{3}HTdR in a 72-hr assay.

[b] Italics indicates cpm 5-fold or greater over background.

TABLE III

Effect of antigen presenting cell population on clone specificity[a]

	Cells		Proliferative Response to Antigen:							
	TLC	APC	Flu A	Flu B	GAT	TGAL	T. Tox.	KLH	Medium	TCGF
			cpm ± SEM							
A	6	E$^-$	*12,661 (239)*[b]	171 (135)	82 (64)	16 (2)	50 (4)	29 (11)	22 (4)	*1,943 (53)*
	24	E$^-$	*7,442 (180)*	35 (27)	14 (3)	62 (2)	10 (2)	19 (4)	17 (4)	701 (141)
	26	E$^-$	*5,656 (830)*	19 (7)	13 (1)	26 (14)	37 (7)	6 (0)	37 (29)	*1,271 (190)*
	37	E$^-$	*6,306 (590)*	289 (57)	41 (27)	20 (0)	57 (25)	46 (9)	15 (1)	1,339 (281)
	71	E$^-$	*2,151 (332)*	18 (8)	10 (12)	30 (4)	37 (11)	420 (188)	27 (1)	*2,117 (490)*
	72	E$^-$	*1,624 (128)*	16 (2)	16 (4)	73 (57)	38 (2)	43 (10)	18 (3)	*404 (8)*
	50	E$^-$	23 (3)	50 (36)	26 (0)	11 (1)	124 (34)	39 (3)	202 (184)	*1,872 (720)*
B	53	–	17 (9)	24 (4)	16 (0)	10 (4)	25 (7)	33 (7)	28 (2)	*1,366 (94)*
	53	PBL	*8,310 (553)*	59 (27)	22 (6)	33 (15)	*610 (117)*	47 (10)	19 (5)	–
	53	E$^+$	32 (10)	24 (3)	49 (3)	10 (0)	39 (8)	37 (1)	22 (2)	–
	53	E$^-$	*5,542 (590)*	26 (7)	51 (47)	25 (1)	59 (14)	43 (12)	19 (3)	–
	53	E$^+$ + E$^-$	*8,384 (991)*	86 (6)	24 (16)	48 (13)	*781 (195)*	37 (3)	11 (3)	–

[a] A. 5 × 10^3 T cells from TLC were stimulated with influenza A, influenza B, GAT, TGAL, T. Tox, or KLH together with 5 × 10^3 autologous irradiated sheep erythocyte rosette negative (E$^-$) cells. B. 5 × 10^3 T cells from TLC 53 were cultured with influenza A, influenza B, GAT, TGAL, T. Tox, or KLH in the presence of 25 × 10^3 autologous irradiated E$^-$ cells or 20 × 10^3 E$^+$ together with 5 × 10^3 E$^+$ cells as a source of antigen presenting cells (APC). Stimulation of both irradiated and unirradiated APC fractions by antigens alone was less than 100 cpm. Incorporation of ^{3}HTdR was measured at 72 hr.

[b] Positive responses italicized.

influenza virus and APC, although it did respond to TCGF. TLC cultured with incompatible APC from panel members in the absence of virus gave responses of less than 50 cpm (data not shown).

Subcloning. To ensure that the properties of the TLC analyzed in this study were those of true clones, TLC 6 was plated in Terasaki trays at 0.3 cells/well in the presence of autologous feeder cells, influenza A virus, and 20% TCGF. The subclones of TLC 6 were expanded and subsequently characterized. Representative subclones (TLC 6.1, 6.3, 6.4, 6.7) possessed identical antigenic specificity and *HLA-D* region restriction between T cell clone and APC as observed with the parent clone TLC 6 (Table V). All subclones responded in the presence of APC to influenza A but not the unrelated antigens, influenza B, GAT, TGAL, T. Tox., or KLH (Table V–A). Furthermore, the presentation of specific antigen observed in the reduced panel of APC of related and unrelated *HLA-D* region phenotypes was identical for the subclones and parent cell in that restriction to *HLA-DW1:DR1* was observed (Table V–B). Control responses of TLC 6 and its subclones to APC in the absence of virus was <30 cpm and confirmed that the response was not due to stimulation by alloantigens.

Immunofluorescence analysis of TLC phenotype. The phenotypes of TLC 6 and 37, which had been maintained in continuous culture for 5 mo, were characterized using a panel of monoclonal anti-T cell antibodies (Table VI). Both clones

TABLE IV

Genetic restriction of virus induced proliferation of TLC to HLA-D/DR antigens[a]

Presenting Cell Panel	HLA-D Region Phenotype		Clones				
	DW	DR	6	37	53	71	50
LNAT[b]	1/3	1/3	*27,951 (2,520)*[c,d]	*6,285 (1,024)*	*4,568 (826)*	*849 (37)*	40 (14)
MLAU	1/1	1/1	*42,782 (941)*	*9,237 (2,034)*	*10,952 (2,070)*	*2,520 (54)*	nt[e]
GARD	1/1	1/1	*11,547 (1,012)*	*3,831 (571)*	*4,001 (628)*	*436 (12)*	nt
JSAU	1/3	1/3	*16,703 (3,147)*	*87,721 (1,547)*	nt	24 (4)	100 (18)
JLMB	1/10	1/4	*14,076 (3,190)*	*1,719 (17)*	*5,904 (2,678)*	50 (22)	24 (12)
EKET	1/?	1/5	*26,552 (387)*	*17,097 (2,475)*	*13,135 (1,014)*	55 (10)	15 (5)
GHOV	3/3	3/?	24 (4)	15 (3)	29 (3)	20 (6)	23 (16)
BMIN	3/?	3/?	292 (242)	391 (83)	nt	31 (5)	nt
OSMO	3/5	3/5	315 (177)	66 (4)	271 (67)	*5,281 (858)*	40 (11)
RKEL	3/6	3/w6.1	23 (1)	22 (10)	nt	173 (71)	32 (4)
KCAR	2/2	2/?	13.(1)	19 (1)	nt	28 (12)	17 (1)
RCAR	2/7	2/7	17 (4)	17 (1)	nt	16 (2)	61 (37)
DBUD	4/DB3	4/?	25 (6)	12 (0)	nt	*1,374 (172)*	37 (9)
		Ag alone	34 (4)	21 (4)	28 (4)	21 (1)	17 (2)
		Medium	38 (6)	27 (6)	27 (5)	28 (6)	17 (3)
		TCGF	*4,766 (256)*	*3,187 (208)*	*2,425 (121)*	*5,339 (333)*	*1,773 (273)*

[a] 5×10^4 TLC cells were stimulated with 5 HA units/ml of influenza A virus in the presence of 5×10^4 irradiated E^- cells from a panel of HLA-D region related and unrelated donors. TLC cultured with APC in the absence of influenza virus gave responses less than 60 cpm. Stimulation was assessed in a 72-hr ^{3}HTdR incorporation assay.

[b] Autologous control from which TLC were derived.

[c] Values expressed as mean cpm (± SEM) of triplicate cultures.

[d] Italicized values indicate positive responses.

[e] Not tested.

TABLE V

Antigen-specificity and MHC restriction of subclones of TLC 6[a]

	Antigen	Presenting cell panel	HLA-DR phenotype		Clone[b,c]	Subclones			
			DW	DR	6	6.1	6.3	6.4	6.7
A.	Flu A	LNAT[d]	1/3	1/3	*13,372 (1,262)*	*11,786 (555)*	*12,042 (898)*	*13,285 (698)*	*14,405 (289)*
	Flu B	–			19 (5)	15 (2)	17 (1)	14 (6)	27 (5)
	GAT	–			19 (4)	23 (4)	18 (2)	18 (3)	12 (3)
	TGAL	–			31 (5)	29 (12)	26 (6)	23 (4)	17 (2)
	T. Tox	–			28 (10)	23 (2)	15 (2)	25 (4)	33 (10)
	KLH	–			17 (1)	19 (3)	15 (4)	15 (1)	14 (2)
B.	Flu A	LNAT	1/3	1/3	*11,947 (755)*	*13,083 (597)*	*11,330 (105)*	*12,071 (255)*	*13,921 (999)*
	–	–			25 (4)[e]	11 (5)	17 (1)	19 (3)	15 (7)
	–	MLAU	1/1	1/1	*22,569 (1,990)*	*19,199 (1,024)*	*19,287 (820)*	*26,083 (299)*	*22,917 (359)*
	–	–			24 (2)	17 (4)	15 (4)	19 (2)	17 (2)
	–	JLMB	1/10	1/4	*5,895 (281)*	*5,943 (450)*	*5,607 (354)*	*4,788 (209)*	*5,021 (125)*
	–	–			15 (5)	19 (3)	21 (5)	17 (3)	13 (3)
	–	EKET	1/?	1/5	*12,319 (1,095)*	*13,098 (896)*	*11,931 (1,137)*	*10,736 (489)*	*13,007 (967)*
	–	–			13 (4)	13 (2)	17 (6)	16 (2)	17 (2)
	–	GHOV	3/3	3/?	22 (2)	12 (5)	13 (3)	15 (2)	17 (5)
	–	–			14 (2)	22 (3)	16 (1)	19 (2)	11 (1)
	–	RCAR	2/7	2/7	32 (4)	14 (2)	11 (4)	23 (7)	17 (1)
	–	–			15 (2)	14 (0)	13 (2)	19 (4)	13 (2)
	–	RKEL	3/6	3/w6.1	17 (6)	17 (3)	15 (5)	18 (4)	13 (1)
	–	–			13 (2)	21 (3)	15 (3)	10 (3)	16 (3)
				Ag alone	19 (1)	15 (1)	11 (4)	23 (7)	17 (1)
				Medium	21 (4)	10 (3)	13 (4)	14 (2)	21 (7)
				TCGF	*5,732 (746)*	*8,217 (282)*	*7,141 (798)*	*6,697 (1,120)*	*8,279 (341)*

[a] A. 5×10^4 T cells from TLC 6 or subclones 6.1, 6.3, 6.4, 6.7 were stimulated with specific and unrelated antigen in the presence of 5×10^4 irradiated autologous E^-. B. 5×10^4 T cells from TLC 6 or subclones 6.1, 6.3, 6.4, 6.7 were stimulated with influenza A virus in the presence of 5×10^4 irradiated E^- cells from HLA-D region related and unrelated donors. Stimulation was assessed with 72-hr ^{3}HTdR incorporation assay.

[b] Values expressed as mean cpm (± SEM) of triplicate cultures.

[c] Italicized values indicate positive responses.

[d] Autologous control from which TLC were derived.

[e] Response of TLC and E^- cells culture in the absence of influenza A virus.

TABLE VI

Phenotypic analysis of TLC

Monoclonal-Antibody	Clone No.	
	6*	37*
	%	
HLA framework	100	100
DR	100	100
OKT3	80	75
OKT4	85	75
OKT6	<1	<1
OKT8	<1	<1
Leu 1	90	3
Leu 2A	<1	<1
Leu 3A	80	100
sig	<1	<1

* Each TLC formed >95% E-rosettes.

were positive with OKT3 (pan-T cell) as well as for HLA- and DR-framework antigens. Furthermore, both TLC were reactive with OKT4 and Leu 3A, which detect an inducer/helper marker. Cells from neither clone were stained by OKT6 (thymocytes), OKT 8, or Leu 2A (suppressor/cytotoxic cells) or anti-surface Ig (B cells). Interestingly, Leu 1, a pan-T-reagent which is similar to OKT3, but which detects a different cell surface molecule, was found on TLC 6 but not on TLC 37. Each TLC formed more than 95% E-rosettes.

DISCUSSION

The *in vitro* generation of discrete subsets of human T cells with different functions, such as help, suppression, cytotoxicity, and proliferation, has been reported for a variety of antigens (27, 34–36). Furthermore, it is known that long-term cultures of human T cells with specificity for allogeneic (25, 28) or soluble antigens (22–24) can be maintained using TCGF. In the present study we report the generation, maintenance, and characterization of cloned human T cells that proliferate specifically in response to influenza A virus.

The unfractionated PBL from a high responder to influenza A virus were cultured with a single pulse of influenza A virus to induce blast transformation of T cells. These cells were subsequently cloned by limiting dilution, thus making it possible to select for antigen specificity before clonal expansion (25–27). Murine (18) and human (24) proliferating T cell clones, selected for antigen specificity before clonal expansion in TCGF, have also been reported, whereas other techniques have centered on enriching for specific cells by repeated antigenic stimulation and culture in the presence of TCGF (14, 15). A potential problem with the latter approach is that the cells that grow well *in vitro* in response to TCGF are not necessarily those cells that are functionally significant *in vivo*.

In the studies reported here, the frequency of growing clones after plating at one cell every three wells was approximately 35%. Different plating efficiencies have been reported by different investigators in a variety of systems (23, 25). Such differences may reflect the nature of the antigens used, the presence of both specific and nonspecific recruitable T cells, quantitative or qualitative differences in the T cell subsets found in peripheral blood compared with lymphatic organs, or merely variations in technical procedures. From the onset, there were concerns as to whether or not these cells were clones. By Poisson probabilities, 99% of the wells should have contained one or fewer cells, and the functional data obtained were consistent with this observation. However, by subcloning and analyzing the antigenic specificity and MHC restriction of both the subclones and the parent cell line as shown in Table V, the true clonal nature of the TLC could be examined. The reaction of TLC 6 subclones, as anticipated, was absolutely identical with that of the parent clone.

The stimulation of the influenza virus-induced clones with a panel of antigens resulted in significant proliferation of the clone in response to the inducing antigen. Additionally, at least one other unrelated antigen always restimulated when irradiated autologous PBL were used as a source of APC. In an attempt to resolve this problem, different fractions of PBL were assayed for their ability to act as APC. It was observed that the addition of an irradiated E^- fraction consisting primarily of B cells and monocytes resulted in the proliferation of TLC when stimulated with influenza A, but not with unrelated antigen. Thus, one likely explanation for the apparent cross-reactivity of influenza A-specific TLC was that it was due to the presence of an irradiation-resistant T cell population that upon antigen stimulation released TCGF or other growth promoters, which in turn induced nonspecific proliferation ("back stimulation") of the TLC. This is supported by the report that irradiation-resistant T cells can release TCGF after mitogen stimulation (28). Thus, all of the reactive clones analyzed in this report were antigen specific, in that they responded to influenza virus strain A, the inducing the antigen, but not to influenza strain B or other unrelated protein antigens. This confirms two previous observations of antigen-specific human T cell clones (23, 24). The detailed analysis of the viral recognition pattern of the influenza virus-immune TLC is reported elsewhere.[6]

For the majority of TLC tested, induction of antigen-specific proliferation required that the APC and the responding TLC share *HLA-D* region specificities. This was documented in experiments in which recognition of antigen by three of five TLC was restricted to those APC from unrelated individuals bearing *Dw1*;*DR1* antigens and confirmed for the subclones of TLC 6. These particular results confirm the findings of many laboratories (22, 24, 26, 27). However, in contrast to the other clones, antigen presentation to TLC 71 was not restricted by any obvious *HLA* antigens and suggests that the human *HLA-D* region may show complexity analogous to that of the murine Ia-region provided that many clones are assayed on large enough panels of presenting cells.

In contrast to other reports (18, 24) TCGF is used in our system only for expanding TLC cultures and is not required during proliferation assays. Interestingly, murine cytotoxic clones require the continuous presence of both TCGF and feeders for proliferation (37). Furthermore, the T-helper cell, which is capable of inducing cytotoxic T cell proliferation, appears to be restricted by class II *Ia* region antigens (38, 39). Thus, human T cell clones that show TCGF dependency as well as antigen specificity may be cytotoxic cells or their precursors.

To determine with which subset of T lymphocytes the influenza virus-specific TLC were associated, by their cell-surface phenotypes, were analyzed using monoclonal anti-human T cell reagents (4, 5). Two clones, TLC-6 and 37, which had been maintained in continuous culture for 5 mo, were characteristic of activated T cells in that they were $OKT3^+$, DR^+, and surface Ig^-. Furthermore, they appear to belong to the inducer/helper subset as opposed to the suppressor/cytotoxic cell subset, in that they were $OKT4^+$, $Leu3A^+$, $OKT8^-$, and $Leu2A^-$. Kurnick *et al.* (23) have reported that antigen-specific T cell clones that proliferate strongly and cooperate with B

[6] Lamb, J. R., D. D. Eckels, M. Phelan, J. N. Woody, and P. Lake. Antigen-specific human T lymphocyte clones: Viral specificity of influenza virus immune clones. Submitted for publication.

cells in the production of antibody are also OKT4$^+$.

Finally, human antigen-specific T cell clones are a powerful tool and should allow the detailed analysis of many functional aspects of lymphocyte interaction and elucidation of the fine specificity of genetically restricted responses previously impossible with conventional cell-culture techniques.

Acknowledgments. The authors thank Ms. E. Ketterer and Ms. D. Hartman for expert technical assistance, as well as Ms. D. Sykes and Ms. B. Minor for secretarial help. Special thanks are due to Drs. J. Neefe and A. Fauci for critical review of this manuscript.

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0022-1767/82/1283-1428$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 128, No. 3, March 1982
Printed in U.S.A.

ANTIGEN-SPECIFIC HUMAN T LYMPHOCYTE CLONES: VIRAL ANTIGEN SPECIFICITY OF INFLUENZA VIRUS-IMMUNE CLONES[1]

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Human T lymphocyte clones (TLC) specific for type A (A/Texas/1/77) influenza virus and maintained in continuous culture with T cell growth factor, were analyzed to define the cellular specificity pattern of virus recognition. A panel of TLC were stimulated with strains of serologically characterized type A influenza subtypes. Five TLC recognized all the viral subtypes; the remaining clones recognized only subtypes that shared serologically defined determinants with the immunizing subtype. In addition, the 11 TLC were analyzed for their fine antigenic specificity by using the purified viral components hemagglutinin (HA), neuraminidase (NA), matrix protein (MP), and nucleoprotein (NP). Five TLC proliferated in response to NA, four to MP, one to HA, and one to NP. None of the clones responded to the unrelated B strain influenza virus, B/Singapore. Furthermore, the fine specificity of an MP-reactive TLC was confirmed by subcloning.

Infection of mice with type A influenza virus can prime both humoral and cell-mediated immunity directed at other subtypes within the strain, a phenomenon termed heterotypic immunity (1–3). Furthermore, antibody responses to influenza virus vaccines are enhanced by previous infection with heterotypic influenza viruses as shown in hemagglutination inhibition (HI)[5] assays (1, 4). Such cross-protection was demonstrated for influenza A subtypes with unrelated hemagglutinin (HA) and neuraminidase (NA) subunits, but not between strain A and strain B influenza viruses. As HA is a T-dependent antigen (5), this suggests that a hapten-carrier effect (6, 7) may occur in the production of specific antibody. This is supported by recent reports that T cells that recognize matrix protein (MP), an internal component of the influenza virion, can cooperate with B cells that recognize HA to enhance the HI antibody response (8, 9). The internal antigens MP, nucleoprotein (NP), and P proteins of various influenza A strain subtypes are cross-reactive (10), although minor differences in their antigenicity have been reported (11). The possibility that these common proteins shared antigenic determinants recognized by T helper cells may help to explain the mechanisms that underlie heterotypic immunity.

The viral specificity of cytotoxic T cell responses to influenza virus has been analyzed in detail (2, 3, 12–18, reviewed in 19). Immunization of mice with subtypes of influenza A virus generates two distinct subpopulations of H-2-restricted cytotoxic T lymphocytes. One population was specific for the immunizing virus (12, 13), recognizing the HA subunit (14, 15); the other exhibited a high degree of cross-reactivity with other subtypes by virtue of its specificity for MP (16, 17). However, the cross-reactivity was restricted to strain A subtypes, and no reaction with strain B influenza virus was observed (13). Recent analysis of cloned lines of murine influenza virus-specific cytotoxic T lymphocytes demonstrated recognition patterns that were either specific for the immunizing virus, subtype-specific in that they reacted with determinants common to viruses of the same subtype as the immunizing virus, or were cross-reactive with all influenza A subtypes (18). In man, the secondary response of a polyclonal population of cytotoxic T cells to influenza A viruses has been directed against cross-reactive determinants of the strain A subtypes expressed on the target (20).

Recently we described the induction and properties of influenza virus-immune human T lymphocyte clones (TLC) (21), the genetic requirements for antigen presentation to TLC, and here we report the viral recognition pattern of these clones determined by proliferation assays. Two major groups of TLC were observed, one that recognizes antigenic determinants common to all influenza A viruses and the other that responds only to determinants within a given subtype. The precise nature of the antigens recognized by the TLC was determined by their responses to purified viral components.

MATERIALS AND METHODS

Cells. Mononuclear cells from peripheral blood (PBL) were obtained from a healthy adult donor and separated by flotation on Ficoll-Hypaque (22) and cryopreserved as previously described (21).

Antigens. The influenza type A viruses used for this study were: A/Puerto Rico/8/34 (H_1N_1), A/Swine/76/31 (H_1N_1), A/Omachi/1/53 (H_1N_1), A/USSR/92/77 (H_1N_1), A/FW/1/50 (H_1N_1), A/Fukushima/103/78 (H_1N_1), A/Japan/305/57 (H_2N_2), A/Japan/170/62 (H_2N_2), A/Taiwan/1/64 (H_2N_2), A/Udorn/304/72 (H_3N_2), A/Port Chambers/1/73 (H_3N_2), A/Victoria/3/75 (H_3N_2), A/Texas/1/77 (H_3N_2), A/Bangkok/1/79 (H_3N_2) and B/Singapore/222/79. The viruses were grown in the allantoic cavity of embryonated chicken eggs. Immunochemically purified HA (A/Bangkok/1/79), NA (Papau New Guinea/1/75), and MP (A/Bangkok/1/79) were generously provided by Dr. R. G. Webster, St. Juce Children's Research Hospital, Memphis, TN. Purified NP (A/Hong Kong/X31) was the kind gift of Dr. J. J. Skehel, National Institute for Medical Research, Mill Hill, London, UK.

Received for publication October 2, 1981.
Accepted for publication December 15, 1981.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] This study was supported by funds received from the Office of Naval Research Contract N000-14-77-C-0748 Research Task No. NR 207-102 and Contract N000-14-77-0747 Research Task No. NR 207-101. The opinions herein are those of the authors and are not to be construed as those of the Department of the Navy or the Department of Defense.
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[5] Abbreviations used in this paper: AET, S-2-Aminoethylisothiouronium bromide hydrobromide; HA, hemagglutinin; HAU, hemagglutinating units; HI, hemagglutination inhibition; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; E^+, sheep erythrocyte rosette-positive mononuclear cells; E^-, sheep erythrocyte rosette-negative mononuclear cells; MP, matrix protein; NA, neuraminidase; NP, nucleoprotein; TCGF, T cell growth factor (interleukin 2); TLC, T lymphocyte clone; ^{3}H-TdR, ^{3}H-thymidine.

T cell growth factor (TCGF). PBL were cultured at 1 × 10⁶/ml in RPMI 1640 medium supplemented with 2 mM L-glutamine, 50 μg/ml gentamicin, 25 mM HEPES* buffer, 25 IU/ml sodium heparin, 1% autologous plasma, and 0.1% v/v phytohemagglutinin-P (PHA-P; Difco Laboratories, Detroit, MI; 22). The supernatants were harvested after 48 h, passed through 0.22-μm filters, and assayed for their ability to support the growth of a TCGF-dependent line as determined by tritiated thymidine (^{3}H-TdR) incorporation. Acceptable lots of TCGF were stored at 4°C and were used as required.

Fractionation of lymphocyte populations by sheep erythrocyte rosetting. Erythrocyte (E) rosettes were formed with sheep red blood cells (SRBC) treated with AET (S-2-aminoethylisothiouronium bromide hydrobromide, Calbiochem, San Diego, CA) as described (21). One volume of packed SRBC was incubated with 5 vol of AET (40.2 mg/ml in distilled H_2O, pH 9.0) for 20 min at 37°C. One volume of PBL at 10^7 cells/ml was mixed with 2 vol of 2% AET-SRBC and 0.5 vol of fetal calf serum. The suspension was centrifuged at 250 × G for 10 min and was placed on ice for 60 min. After resuspension of the pellets by gentle rotation, the separation of E rosette-forming (E^+) from nonrosetting (E^-) lymphocytes was achieved by centrifugation (1500 × G, 15 min) over Percoll (ρ = 1.080 g/ml; Pharmacia, Piscataway, NJ). The E^- cells were recovered from the interface and contained less than 1% E^+ cells. In proliferation assays, cultures were reconstituted with 5 × 10^3 E^+, as a source of antigen-presenting cells.

Induction and assay of antigen-specific T lymphocyte clones (TLC). Human T cells specific for influenza A virus were induced as described (21). Briefly, 10^6 PBL/ml were cultured in medium supplemented with 10% A^+ serum and 5 hemagglutinating units (HAU)/ml of attenuated influenza virus (A/Texas/1/77). After 6 days, responding cells were enriched on a 35 to 40% Percoll gradient, resuspended at a concentration of 33 ½ cells/ml in medium containing 20% TCGF, and were plated at one cell every third well in Terasaki trays with 10^4 irradiated autologous PBL and the optimal concentration of influenza A virus. After 7 days, growing clones were transferred to 96-well flat-bottomed trays and subsequently to 24-well plates and were then expanded in 25-cm² tissue culture flasks. Subclones were induced by using the same initial cloning procedure, namely TLC cells were plated at 0.3 cells/well in Terasaki plates with irradiated autologous PBL and influenza virus in the presence of 20% TCGF. The cultures showing positive growth at 7 days were expanded as already described. Fresh 20% TCGF was added every 3 to 4 days and irradiated pooled feeders (5 × 10^5/ml) were added every 7 days. Before use in proliferation assays, TLC were rested 6 to 7 days after the addition of feeders.

TLC were examined for antigen-specific proliferation in a 72-h assay by using ^{3}H-TdR incorporation. Five thousand TLC were cultured with irradiated E^- cells (5 × 10^4) and antigen in a total volume of 200 μl. After 72 h, cultures were pulsed with 1.0 μCi of ^{3}H-TdR and were harvested 8 to 16 h later. Incorporation of radiolabeled thymidine was measured by liquid scintillation spectroscopy. Results are expressed as the mean counts per minute (cpm) ± 1 standard error of the mean (SEM) for triplicate cultures or as the percent of the response to the immunizing virus.

RESULTS

Pattern of influenza virus recognition exhibited by virus-specific TLC. A panel of 11 TLC, selected for their ability to proliferate in response to the inducing antigen (A/Texas/1/77, H_3N_2), were cultured with irradiated E^- lymphocytes, as a source of antigen-presenting cells, in the presence of appropriate virus strains representative of the major type A influenza virus subtypes. Also included were swine influenza (A/Swine/76/31, H_1N_1) and the unrelated influenza B strain, B/Singapore/222/79. Table I shows the results of this analysis. Of the 11 TLC induced and selected with A/Texas/1/77, only one clone (TLC 11) proliferated when cultured only with the H_3N_2 subtypes (A/Udorn/304/72; A/Port Chambers/1/73; A/Victoria/3/75; and A/Bangkok/1/79). This result suggests that TLC 11 recognizes an antigenic determinant on the HA molecule shared by the H_3N_2 subtypes. Five TLC (26, 53, 57, 71, and 95) proliferated in response to all H_3N_2 and H_2N_2 subtypes but failed to proliferate when cultured with any of the H_1N_1 subtypes (A/PR/8/34; A/Swine/1976/31; A/FW/1/50; A/Omachi/1/53; A/USSR/92/77; and A/Fukushima//103/78). Thus, TLC 26, 53, 57, 71, and 95 were specific for NA; only A strain subtypes bearing N_2 were able to stimulate these five clones. The remaining five clones (TLC 6, 37, 39, 72, and 83) proliferated in the presence of viruses representative of all strain A influenza virus subtypes. Therefore, these TLC recognize an antigen such as MP or NP that is shared by all the subtypes. None of the TLC analyzed showed antigenic specificity restricted to only the inducing subtype A/Texas/1/77) and they were unresponsive to strain B influenza virus (Table I). Also, in the absence of E^- antigen-presenting cells, none of the TLC responded to any of the virus strain A subtypes (data not shown).

Reactivity of virus-specific TLC with influenza virus components. The same panel of 11 TLC was analyzed for reactivity with influenza virus components (Table II). Each TLC was stimulated with the purified components, HA (0.1 μg/ml), NA (10^{-3} v/v), MP (0.1 μg/ml), and NP (0.1 μg/ml). The antigens were used at the optimal concentration for inducing maximum proliferation of unfractionated PBL of the donor whose cells were cloned. In addition to viral components, the TLC were stimulated with the intact inducing virus (A/Texas/1/77) and the unrelated strain B influenza virus (B/Singapore/222/79).

TLC 11 proliferated when cultured with HA but failed to respond to NA and MP. Although the MP preparation may contain residual HA (R. G. Webster, personal communication), it is clearly not present in sufficient quantities to elicit a response from TLC 11. Five TLC (26, 53, 57, 71, and 95) proliferated only in response to the viral component NA. Of the remaining clones, four (6, 37, 39, and 72) proliferated when

TABLE I

*Recognition pattern of influenza virus strain A subtypes by T lymphocyte clones**

Virus Subtype	H	N	Clone No.										
			11	26	53	57	71	95	6	37	39	72	83
A/Texas/1/77	3	2	100	100	100	100	100	100	100	100	100	100	100
A/Udorn/304/72	3	2	100†	98	102	107	114	122	131	132	111	138	107
A/Pt. Chalmers/1/73	3	2	109	122	113	99	120	109	117	120	103	116	100
A/Victoria/3/75	3	2	129	118	101	91	133	99	123	126	113	94	74
A/Bangkok/1/79	3	2	124	106	117	131	98	103	115	99	98	102	113
A/Japan/305/57	2	2	1	100	121	84	63	98	108	98	99	114	87
A/Japan/170/62	2	2	3	94	92	102	99	109	120	110	102	96	101
A/Taiwan/1/64	2	2	2	108	131	111	100	90	63121	64	85	91	115
A/FW/1/50	1	1	1	1	1	1	2	1	101	72	106	103	84
A/Omachi/1/53	1	1	1	<1	<1	1	2	1	116	76	119	82	100
A/USSR/92/77	1	1	1	2	1	2	1	1	97	60	95	124	108
A/Fukushima/103/78	1	1	1	1	1	1	3	1	93	100	105	69	127
A/PR/8/34	1	1	2	3	1	2	4	1	107	84	110	86	98
A/Swine/1976/31	1	1	2	1	1	3	2	2		90	80	110	78
B/Singapore/222/79			1	<1	<1	<1	<1	<1	1	<1	1	1	1

* A panel of 11 T lymphocyte clones (2.5 × 10^4/ml) was cultured with 16 influenza viruses at final dilutions of 1/1000 and 1/5000 in the presence of autologous irradiated E^+ cells (2.5 × 10^4/ml). Stimulation was assessed by the incorporation of ^{3}H-thymidine in a 72-hr assay.

† Results expressed as the percent proliferation of the mean response of each TLC to A/Texas/1/77. Control responses of each TLC to virus in the absence of E^+ was <50 cpm as was the response to virus of E^+ cells alone.

cultured with MP but not with HA or NA, whereas TLC 83 failed to respond to HA, NA, or MP. However, when cultured with NP, TLC 83 proliferated vigorously (Table II). To ensure that the antigenic specificity of the TLC analyzed in this study were those of true clones, TLC 6 was subcloned by limiting dilution. The specificity of representative subclones (TLC 6.1, 6.3, 6.4, 6.5, and 6.7) was identical to that of the parent TLC 6 in that they responded to MP but not to any of the other influenza viral components tested or to influenza strain B (Table III).

DISCUSSION

The results presented in this report suggest that the specificity of proliferative responses by human T lymphocytes to influenza virus are highly heterogeneous. At the clonal level, it is evident that proliferating clones with several distinct specificities of influenza virus recognition are present. Analysis of the clones on a panel of strain A virus subtypes revealed two patterns of response. Firstly, clones that recognizes all strain A subtypes are evident, and secondly, those that responded to determinants shared by viruses of the same subtype as the immunizing virus are found as well. However, TLC in neither group cross-reacted with influenza B virus.

Similar reports of subtype-specific and cross-reactive populations exist for human cytotoxic T cells primed with type A influenza (20, 24, 25). Our observation that T cell clones recognize specific and shared type A viral antigenic determi-

TABLE II

Specificity of virus-immune T lymphocyte clones to purified viral components[a]

	Clone No.										
	11	28	53	57	71	95	6	37	39	72	83
Influenza A strain (A/Texas/1/77; 5 HAU/ml)	7962[b,c] (400)	7812 (1728)	7419 (705)	8542 (582)	3570 (2018)	6550 (876)	9343 (504)	18191 (479)	5635 (595)	7739 (263)	6358 (1266)
Influenza B strain (B/Singapore; 5 HAU/ml)	37 (14)	16 (1)	47 (6)	23 (4)	24 (8)	14 (5)	45 (8)	20 (2)	28 (2)	21 (1)	37 (15)
Hemagglutinin (0.1 μg/ml)	5467 (925)	19 (1)	54 (21)	10 (2)	28 (14)	17 (4)	70 (30)	41 (5)	43 (20)	17 (2)	16 (2)
Neuraminidase (10^{-3} v/v)	8 (4)	3680 (344)	7123 (709)	4213 (382)	5291 (1249)	4031 (612)	21 (1)	19 (5)	52 (13)	15 (3)	19 (7)
Matrix protein (0.1 μg/ml)	16 (2)	19 (2)	37 (9)	23 (7)	27 (8)	19 (2)	4132 (751)	9752 (1677)	4668 (710)	6666 (777)	21 (3)
Nucleoprotein (0.1 μg/ml)	18 (2)	32 (5)	29 (5)	41 (5)	35 (8)	33 (10)	57 (27)	77 (27)	26 (7)	57 (10)	4581 (539)
Medium	15 (4)	23 (3)	35 (16)	19 (4)	14 (3)	16 (4)	146 (45)	30 (6)	21 (2)	39 (4)	27 (4)
TCGF	4375 (255)	5039 (769)	7283 (281)	3679 (721)	2741 (507)	2387 (37)	4725 (395)	10987 (1159)	3243 (252)	4915 (675)	6695 (126)

[a] Each TLC at 2.5×10^4/ml was stimulated with HA, NA, MP and NP in the presence of 2.5×10^4 autologous irradiated E^+ cells/ml. Stimulation was assessed by ^{3}H-TdR incorporation in a 72-hr assay.

[b] Proliferation expressed as mean cpm (±SEM).

[c] Underline indicates cpm greater than 230-fold over background.

TABLE III

Viral component specificity of subclones of TLC 6[a]

Viral Components	Clone	Subclones				
	6	6.1	6.3	6.4	6.5	6.7
Influenza A strain (A/Texas/1/77; 5 HAU/ml)	18134[b,c] (815)	16187 (127)	13960 (1266)	21092 (198)	16483 (400)	13677 (1222)
Influenza B strain (B/Singapore; 5 HAU/ml)	35 (5)	22 (2)	27 (14)	40 (1)	30 (3)	30 (6)
Hemagglutinin (0.1 μg/ml)	27 (8)	22 (8)	15 (5)	26 (6)	14 (2)	34 (11)
Neuraminidase (10^{-3} v/v)	45 (20)	32 (2)	19 (4)	33 (14)	27 (4)	17 (2)
Matrix protein (0.1 μg/ml)	11560 (407)	12155 (330)	12166 (833)	24953 (825)	12605 (772)	10375 (585)
Nucleoprotein (0.1 μg/ml)	32 (8)	41 (19)	33 (14)	41 (2)	28 (4)	37 (9)
Medium	24 (4)	25 (4)	30 (7)	16 (4)	15 (5)	19 (1)
TCGF	3225 (516)	4222 (276)	7131 (629)	8431 (662)	4068 (127)	4840 (413)

[a] TLC 6 or subclones of TLC 6 at 2.5×10^4/ml were stimulated with HA, NA, MP, and NP in the presence of 2.5×10^4 autologous irradiated E^+ cells/ml. Stimulation was assessed by ^{3}H-TdR incorporation in a 72-hr assay.

[b] Proliferation expressed as mean cpm (±SEM).

[c] Underline indicates cpm greater than 20-fold over background.

nants confirms and extends earlier reports of two distinct populations of influenza-reactive, cytotoxic T cells (12–15). However, none of the TLC reported here proliferated only in response to the inducing virus A/Texas/1/77, findings similar to those reported for cloned mouse lines of anti-influenza-specific cytotoxic T lymphocytes (26). This is contrary to another report of the specificity of cloned mouse lines of influenza virus-specific cytotoxic cells, in which six of 12 clones analyzed appeared specific for only the immunizing subtype (18). This inference, however, was based on specificity analyses in which only two H_2N_2 subtypes were used. Furthermore, the fine specificity of two cytotoxic clones, one specific for the immunizing virus and the other subtype-specific was investigated. It was interesting that both of these clones appeared to recognize viral HA as determined by their reaction with two recombinant virus strains. This may imply that subtype-specific clones may recognize a "common" region of HA. Our failure to observe clones uniquely specific for the priming virus may be due to exposure of the donor to particular viral antigens during a previous natural infection with influenza A virus. Thus it is likely that we have cloned primed T cells *in vivo*. Furthermore, because there is evidence to suggest that the human secondary cytotoxic T cell response to influenza viruses is predominantly directed against cross-reactive determinants (20, 25), cell specific only for the immunizing strain may appear at too low a frequency in the peripheral blood to be cloned.

Whereas the specificity of cytotoxic T lymphocytes has been reported to be directed predominantly against HA (14) and NP (16, 17), the majority of human proliferative TLC responded to either NA or MP. Although cytotoxic T lymphocytes specific for NA have not been demonstrated directly, our evidence that NA can be recognized by T cells suggests this antigen does participate in cell-mediated immune responses. It is possible that cytotoxic cells specific for NA may be present *in vivo*, but at a much lower frequency than killer cells directed at other viral antigens and lower than is observed for proliferating T cells. Indeed, Koszinowski *et al.* (27) described influenza A-specific, cross-reactive, cytotoxic T cells that may recognize NA on target cells fused with mixed influenza/Sendai liposomes lacking MP. Thus, functionally distinct T cell subsets may show biased reactivity to viral components.

That MP determinants are recognized by cross-reactive lymphocytes, as reported previously (3, 16, 17), is somewhat uncertain since analysis of virus-infected targets, using a panel of monoclonal anti-NP antibodies, demonstrated that NP was expressed on the cell surface at concentrations 10-fold higher than those of MP. This implies that cross-reactive killer cells may also recognize NP (28). Alternatively, it has been suggested that cross-reactive cytotoxic T cells recognize the viral glycoproteins (27). The present study shows that MP-specific T cells are a significant component of the influenza-specific, T cell repertoire, and suggests that this antigen may be recognized by other effector cell subsets. The MP-specific TLC reported here may be important in cellular collaboration during production of HA-specific antibodies as has been described for the mouse (3). Such a phenomenon, analogous to the hapten-carrier effect, may be operating in the production of anti-HA antibodies in man.

Detailed analyses of the antigenic specificity of T cell responses to influenza virus have been made with the use of cytotoxic T cells (18, 19, 25, 26). In this report we have analyzed the fine specificity of human influenza A-specific T lymphocyte clones selected for their ability to proliferate. This approach should allow a precise analysis of the interrelationship of viral antigenic sites recognized by accessory, inducer, or suppressor cells and the mechanisms controlling immunogenicity of antigenic determinants recognized by effector T and B cells.

Acknowledgments. The authors thank Ms. E. Ketterer for expert technical assistance and Ms. D. Sykes for secretarial help. Special thanks are due to Drs. R. G. Webster and J. J. Skehel for their generous gifts of viral antigens.

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These cell lines, like CTCL-3 and CTC-16, were established from the peripheral blood of patients with leukaemia or lymphoma involving relatively mature T cells. An example of the immunofluorescence obtained using monoclonal anti-p19 antibody on MT-1 and MT-2 cells and a positive control cell line, clone A9, is shown in Fig. 2.

A specific competition radioimmunoassay has been described for the HTLV core protein, p24[9], which can detect as little as 0.05 ng of p24. This precipitation assay uses ^{125}I-labelled homogeneous HTLV p24 and a hyperimmune rabbit antiserum against detergent-disrupted HTLV. Competition is observed only with proteins from disrupted HTLV or HTLV-producing cells. As shown in Fig. 3*a*, cell extracts from both MT-1 and the HTLV-producing cell line, clone B-2, competed in the HTLV p24 radioimmunoprecipitation assays. The competition with IdUrd-induced MT-1 cells was about 10-fold higher than with untreated MT-1 cells, and the slope and end point of the competition curves with clone B2 and induced MT-1 were the same. The p24 determinants of HTLV and ATLV which are recognized by this antiserum are thus indistinguishable. No competition was observed with extracts from HTLV-negative control cells, which included cultured human normal mature T cells and leukaemic T-cell lines previously shown to be HTLV negative.

The kinetics of induction of expression of p19 and p24 were analysed in parallel after treatment of MT-1 cells with IdUrd for 24 h. As shown in Fig. 3*b*, the induction of both proteins was time-dependent and increased with increasing concentrations of inducer. There was a slight increase in the expression of both p19 and p24 immediately following the 24-h treatment of MT-1 cells with IdUrd. However, a substantial increase in the expression of both proteins was observed 48 h after IdUrd treatment. At the highest IdUrd concentration tested (60 μg ml^{-1}), the p19 expression increased about 8-fold while a 16-fold increase was detected for p24 compared with the untreated control cells. The expression of both these proteins therefore increased simultaneously on IdUrd treatment of MT-1 cells. It is therefore likely that the 'adult T-cell leukaemia-associated antigen' or 'ATLA' in MT cells detected following induction with IdUrd and using natural antibodies in sera of Japanese ATL patients[3], represents one of these previously described HTLV proteins[9,10].

We have previously shown that >90% of tested Japanese patients with ATL also contained antibodies to HTLV p24[4–6]. A subsequent more extensive screen of a large number of sera of Japanese ATL patients has substantiated the earlier finding[28]. In addition, 25% of close relatives of ATL patients and about 10% of individuals in the area endemic for ATL also have antibodies to HTLV p24. In contrast, none of 40 normal random sera from the non-endemic regions of Japan had anti-HTLV p24 antibodies.

We conclude from the data presented here that the antigens reacting with the antibodies in the sera of most Japanese ATL patients are highly cross-reactive with HTLV proteins p19 and p24. Two cell lines from such patients (MT-1 and MT-2), which contain ATLV RNA and proviral DNA[5], also contain viral nucleotide sequences which are virtually indistinguishable from those of HTLV. MT-1 (which produces ATLV[3]) expresses two antigenic determinants indistinguishable from those of HTLV p24 and p19. Virtually all MT-2 cells (which produce ATLV[4]) express HTLV p19. Moreover, three other Japanese ATL cell lines established in our laboratory (M.P. *et al.*, in preparation) and two other similar lines established in Japan (S. Morikawa, personal communication) also express antigenic determinants indistinguishable from those of HTLV p24 and p19. The widespread occurrence of antibodies in the Japanese ATL sera strongly reactive with HTLV p24 further strengthens our conclusion that the retrovirus associated with Japanese ATL is identical or very closely related to previously described strains of HTLV[1,2,18] which have been isolated and characterized from patients in the US with leukaemias and lymphomas involving mature T cells, and establish ATLV within the HTLV group.

We thank M. Jarvis, E. Richardson and E. Shepard for technical assistance. Fixed cells from MT-2 were provided by Dr Y. Hinuma. M.P. was a recipient of the American Cancer Society, Eleanor Roosevelt International Cancer Fellowship awarded by the International Union Against Cancer.

Received 23 July; accepted 26 August 1982.

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Human T-cell clones recognize chemically synthesized peptides of influenza haemagglutinin

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The continual variation in the antigenic structure of the haemagglutinin (HA) molecule is associated with the appearance of new epidemics or pandemics of influenza in immune populations[1]. The detailed analysis of HA structure has provided the molecular basis of this phenomenon[2–4] and while four antibody-binding sites have been suggested[4], the epitopes of HA that are recognized by T cells remain undefined. Using chemically synthesized peptides corresponding to sequences of the HA molecule[5], we have investigated the antigenic determinants recognized by long-term T-lymphocyte lines and clones induced with HA and maintained in T-cell growth factor (TCGF or interleukin-2, IL-2). The data suggest that HA-specific T-cell populations contain the repertoire to respond to all the synthetic peptides analysed. However, when responses are analysed at the clonal level, one peptide located at the carboxy-terminus of the HA1 molecule and discrete from the proposed antibody-binding sites[4] seems to be immunodominant.

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Peripheral blood lymphocytes (PBL) from adult volunteers selected for known responses to strain A influenza virus (A/Texas/1/77; H_3N_2) were cultured with HA isolated from this subtype. After 6 days, cultures were either cloned by limiting dilution in the presence of TCGF, irradiated autologous PBL and specific antigen as previously described[6], or maintained as cell lines. The T lymphocyte clones and lines were maintained in continuous culture for 8 weeks and then assayed for responsiveness to specific antigen in the presence of antigen-presenting cells as determined by tritiated thymidine (^{3}H-TdR) incorporation.

Normal PBL responded to the intact virus and to the HA molecule of A/Texas/1/77, as did long-term influenza and HA lines induced with the same viral subtype (Table 1). The antigenic specificity of these lines was confirmed by their failure to respond to the unrelated strain B influenza virus, B/Singapore/222/79. Both normal PBL and T-cell lines in the presence of antigen-presenting cells responded to each of the synthetic peptides (Fig. 1) tested over a dose range of 0.01–10 μg ml^{-1}. Although these peptides were synthesized according to the sequences of influenza strain X-47 (ref. 7), in most cases they were identical to corresponding regions of A/Texas/1/77 (ref. 8). Both viruses are H3N2, and comparison of their sequences reveals only a few amino acid differences. It is evident that peptide 20, which corresponds to the carboxy-terminus of HA1, induced the maximum proliferative response (Table 1). Interestingly, this peptide is distant from the four proposed antibody-binding sites. Although these sites are able to induce proliferation, it appears that they are not the immunodominant region of the HA molecule for T-cell responses. In comparison, studies on immune responses to myoglobin in mice have demonstrated that the five antigenic sites recognized by antibody are also the primary inducers of T-cell proliferation in cell populations[9,10] and long-term cultures[11]. Furthermore, it was reported that peptides representing B-cell non-antigenic parts of the molecule were unable to induce proliferation[10], which may reflect the fact that regions of myoglobin are phylogenetically conserved and are thus immunologically silent. However, our findings demonstrate that the entire HA molecule as defined by the synthetic peptides used in these studies can induce T-cell proliferation with none of the peptides behaving as nonimmunogens, as was observed in myoglobin[10], or actively inducing suppression, as has been reported for lysozyme[12,13]. Analysis of the activity of smaller peptides of HA, however, may allow such regions to be identified.

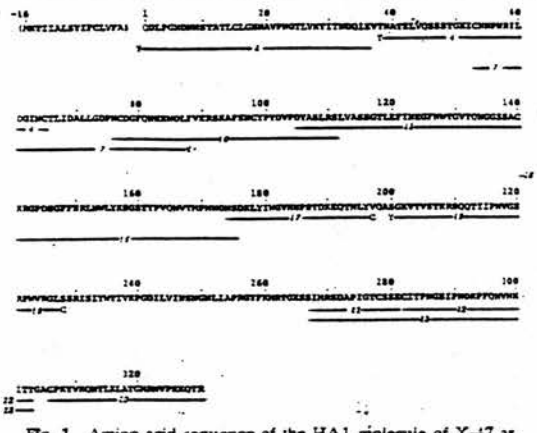

Fig. 1 Amino acid sequence of the HA1 molecule of X-47 as predicted from the nucleotide sequence[7]. The regions of the molecule synthesized are underlined and numbered. C and Y at the end of the peptide indicate, respectively, the addition of a cysteine or tyrosine not present in the primary sequence.

Table 1 Reaction of normal peripheral blood lymphocytes and influenza A virus- and HA-specific long-term T-cell lines with synthetic peptides of the HA1 molecule

Synthetic peptides of HA-1 molecule	Normal PBL	Lymphocytes Influenza A line	HA line
2	2,388 (206)	10,308 (1,737)	1,797 (326)
4	3,461 (171)	1,008 (387)	2,216 (321)
7	6,204 (494)	7,885 (515)	1,497 (296)
10	3,924 (583)	7,287 (1,005)	2,088 (64)
11	5,960 (926)	11,359 (2,179)	1,349 (321)
16	2,359 (639)	9,173 (780)	1,439 (366)
17	1,667 (186)	6,683 (566)	1,470 (264)
19	2,267 (334)	10,091 (25)	1,131 (381)
20	9,061 (1,737)	30,266 (6,240)	3,549 (272)
21	2,460 (373)	5,871 (764)	1,622 (196)
22	4,733 (445)	2,065 (245)	1,378 (174)
23	3,017 (308)	8,743 (952)	2,094 (140)
HA molecule (A/Texas/1/77; H_3)	6,514 (512)	16,792 (1,308)	2,326 (452)
Intact virus (A/Texas/1/77)	16,017 (2,816)	40,645 (9,307)	9,173 (1,072)
Influenza B strain (B/Singapore/222/79)	22,085 (4,969)	57 (10)	27 (16)
Medium alone	21 (5)	20 (6)	21 (6)
TCGF	—	3,481 (365)	1,329 (52)

Normal human peripheral blood lymphocytes (PBL, 2.5×10^5 ml^{-1}) were cultured with either the synthetic peptides of the HA1 molecule, the purified HA molecule (gift of Dr R. G. Webster), or intact influenza A (A/Texas/1/77) or B (B/Singapore/222/79) strain virus for 6 days in RPMI-1640 (Gibco) supplemented with 10% pooled A$^-$ serum. The concentration of antigen used for the synthetic peptides and HA was 0.01–10 μg ml^{-1}, and 0.05–50 haemagglutinating units (HAU) ml^{-1} for the intact viruses. To induce T-cell lines, PBL were cultured with intact A/Texas/1/77 or the purified HA molecule of A/Texas, and after 6 days the lymphoblasts were enriched on a discontinuous Percoll gradient. These blast cells (10^5 ml^{-1}) were expanded as long-term T-cell lines with filler cells (irradiated (2,500 rad) autologous PBL at 5×10^5 ml^{-1}) and specific antigen in the presence of T-cell growth factor. TCGF was prepared by culturing PBL (1×10^6 ml^{-1}) with 0.1% purified phytohaemagglutinin-P (PHA-P; Difco) in RPMI-1640 supplemented with 1% autologous plasma[27]. After 48 h, supernatants were collected and assayed for their ability to support growth of a TCGF-dependent line as described previously[5]. The lines were maintained with filler cells and antigen every 7 days and TCGF every 3 days. Influenza A virus and HA-specific T-cell lines (2.5×10^6 ml^{-1}) were cultured with the panel of antigens in the presence of irradiated autologous PBL (1.25×10^6 ml^{-1}) for 72 h, pulsed for 8–16 h with 1.0 μCi of tritiated methylthymidine (^{3}H-TdR; NEN) and collected on to glass fibre filters. Proliferation, as correlated with ^{3}H-TdR incorporation, was measured by liquid scintillation spectroscopy[5]. Results are expressed as mean c.p.m. (±s.e.m.) of triplicate cultures. Only the highest proliferative response in the antigen dose–response curve is shown. As controls, the responses to medium alone, TCGF and B/Singapore were determined.

The fine specificity of antigen recognition by HA-specific T cells was investigated at the clonal level using chemically synthesized peptides of HA1 (Table 2). One of the four T-lymphocyte clones (HA1.9) proliferated in response to the HA molecule and the intact influenza A virus, but failed to respond to B/Singapore or to the panel of synthetic peptides. Presumably, this clone recognizes determinants that reside in the sequences not covered by overlapping peptides (Fig. 1). The remaining three clones analysed (HA1.4, HA1.7 and HA2.43) responded only to peptide 20 and not to the predominant antibody-binding sites. Comparable to this observation is a report on the clonal analysis of T-cell recognition of the antigenic determinants of myoglobin, where two clones specific for the fragment 1–55 failed to respond to peptide 15–22, which contains the only antibody-binding site[11].

To define the epitope specificity of T-cell antigen recognition, clones HA1.4, HA1.7 and HA2.43 were cultured with the intact viruses A/Japan/305/57 (H2N2) and A/Texas/1/77 (H3N2). Neither clone HA1.4 nor HA1.7 responded to A/Japan, but both proliferated in response to stimulation with A/Texas (Table 3), suggesting that these two clones recognize a variant region of the carboxy-terminus of HA1. Any one of the amino acid substitutions between A/Japan and A/Texas occurring within the sequence of peptide 20 at residues 312–314, 316, 321 and 326–329 (Fig. 2) would be able to confer the specificity of these clones for A/Texas as opposed to A/Japan, as it has been reported that T-cell activation may

Table 2 Recognition of synthetic peptides by HA-specific human T-lymphocyte clones

Synthetic peptides of HA1 molecule	Clone no. HA1.4	HA1.7	HA1.9	HA2.43
2	61 (5)–92 (16)	17 (6)–59 (13)	25 (11)–57 (15)	17 (5)–57 (14)
4	24 (2)–54 (14)	17 (1)–31 (11)	25 (5)–37 (9)	49 (9)–81 (9)
7	33 (7)–136 (50)	13 (4)–38 (1)	16 (8)–51 (19)	10 (1)–61 (7)
10	31 (9)–96 (72)	27 (3)–38 (5)	26 (9)–52 (14)	28 (4)–74 (9)
11	15 (3)–47 (5)	19 (5)–81 (25)	14 (3)–37 (1)	17 (4)–37 (9)
16	16 (4)–65 (45)	15 (4)–26 (10)	14 (8)–93 (51)	15 (4)–43 (8)
17	24 (5)–40 (8)	20 (5)–45 (23)	20 (5)–45 (23)	18 (7)–19 (2)
19	27 (5)–51 (4)	27 (5)–67 (14)	14 (2)–49 (12)	31 (5)–61 (14)
20	69 (46)–3,591 (22)	25 (4)–2,846 (158)	33 (15)–83 (23)	886 (30)–13,662 (1,514)
21	21 (2)–48 (2)	22 (2)–44 (15)	16 (4)–43 (7)	17 (2)–31 (6)
22	18 (4)–44 (7)	19 (4)–39 (7)	16 (2)–28 (4)	11 (3)–21 (3)
23	12 (1)–22 (3)	48 (9)–63 (6)	21 (3)–52 (16)	9 (2)–39 (3)
HA molecule (A/Texas/1/77: H_1)	2,607 (282)	3,073 (180)	10,440 (566)	12,038 (280)
Intact virus (A/Texas/1/77)	7,772 (732)	3,009 (99)	22,389 (6,751)	14,401 (926)
Influenza B strain (B/Singapore/222/79)	37 (11)	29 (2)	43 (6)	18 (2)
Medium alone	20 (1)	27 (4)	27 (4)	29 (2)
TCGF	3,813 (366)	1,018 (29)	8,099 (521)	1,276 (25)

HA-specific human T-lymphocyte clones were isolated as described previously for influenza A virus[5]. Briefly PBL were cultured for 6 days with 0.1 μg ml^{-1} HA. The lymphoblasts enriched on a discontinuous Percoll gradient and resuspended at 33½ cells ml^{-1} in RPMI–1640 supplemented with 10% A$^+$ serum and 20% TCGF, were plated out at one cell every third well in Terasaki trays with 10^4 irradiated autologous PBL and 0.1 μg ml^{-1} HA. After 7 days, growing clones were transferred to 96-well microtitre trays and then to 24-well trays. Then they were expanded in 25-cm^2 tissue culture flasks. At each transfer the T-lymphocyte clones received fresh TCGF and irradiated autologous PBL, together with specific antigen. The clones were maintained with 20% TCGF every 3–4 days and irradiated autologous PBL and HA were added every 7 days. Before use in proliferation assays, the clones were allowed to rest for 6–7 days after the addition of filler cells. HA-specific clones (2.5 × 10^4 ml^{-1}) were cultured with the synthetic peptides of HA1 molecule (0.01–10 μg ml^{-1}), HA molecule (0.01–10 μg ml^{-1}), A/Texas/1/77 (0.05–50 HAU ml^{-1}) or B/Singapore/222/79 (0.05–50 HAU ml^{-1}) in the presence of irradiated autologous sheep erythrocyte rosette-negative (E$^-$) cells (2.5 × 10^4 ml^{-1}) for 72 h, and proliferation measured by ^{3}H-TdR incorporation. E$^-$ cells were fractionated as described previously[5]. Results are expressed as mean c.p.m. (±s.e.m.) of triplicate cultures. Both the lowest and highest proliferative responses for the antigen concentrations used are shown for the synthetic peptides.

Table 3 Specificity of T-cell clones for A/Japan/305/57 (H2N2) and A/Texas/1/77 (H3N2) subtypes of influenza A virus

Antigen	Clone no. HA1.4	HA1.7	HA2.43
Peptide 20	1,145 (138)–15,265 (1,356)	1,047 (207)–17,408 (1,799)	620 (65)–3,373 (481)
A/Texas/1/77	645 (134)–15,561 (438)	975 (251)–17,406 (822)	472 (109)–3,089 (396)
A/Japan/305/57	55 (29)–91 (21)	63 (49)–123 (16)	274 (28)–2,620 (229)
B/Singapore/222/79	19 (5)–42 (6)	25 (3)–56 (2)	27 (3)–49 (9)
Medium alone	21 (3)	55 (23)	11 (3)
TCGF	1,409 (24)	1,982 (57)	1,472 (139)

HA-specific clones (2.5 × 10^4 ml^{-1}) were cultured with peptide 20 (0.01–10 μg ml^{-1}), A/Texas/1/77, A/Japan/305/57 or B/Singapore/222/79 in the presence of irradiated autologous E$^-$ cells (2.5 × 10^4 ml^{-1}) for 72 h. The intact influenza viruses were added at concentrations of 0.05–50 HAU ml^{-1}. Proliferation was determined by the incorporation of ^{3}H-TdR. Results are expressed as described in Table 2 legend.

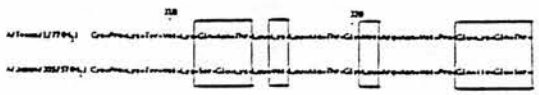

Fig. 2 Amino acid substitutions between A/Japan/305/57 and A/Texas/1/77 occurring within the sequence of peptide 20. The sequence comparison was derived from Gething *et al.*[5]. The sequence numbering is according to Min Jou *et al.*[7] in order to conform with our peptide designations. The boxed areas indicate substitutions.

be sensitive to a single amino acid substitution[14,15]. In contrast to clones HA1.4 and HA1.7, clone HA2.43 recognized both viral subtypes, which implies that it recognizes an invariant sequence of peptide 20, or less likely that the amino acid substitutions between A/Japan and A/Texas are insufficient to cause modification of the molecule to influence T-cell antigen recognition[14].

The findings reported here do not resolve the issue of the nature of T-cell recognition in terms of a defined amino acid sequence or its dependency on tertiary structure[16]. In an attempt to define the epitope sequence, subunits of peptide 20 were generated with the four carboxy-terminal amino acids and increasing the subunit sequence progressively by four amino acids until the complete peptide was synthesized. Unlike the intact peptide, however, none of the subunits induced proliferation of the clones (data not shown). This result does not distinguish whether the amino terminus of peptide 20 is the precise sequence recognized or whether it dictates the conformation of the native peptide necessary for T-cell recognition. An alternative explanation is that the subunits themselves cannot adopt the same conformation as in the intact peptide, therefore they are no longer recognized by the T cells.

The technical assistance of Ms B. Ketteter is acknowledged. This work was supported by the Office of Naval Research Contracts NOOO-14-77CO748, 77CO747 and 80KO909 and the Naval Medical Research and Development Command Work Unit MOO95PN001. We thank Dr R. A. Lerner for helpful discussions in the preparation of this manuscript, which is publication no. 2783 from the Research Institute of Scripps Clinic.

Received 2 August; accepted 2 September 1982.

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Linkage relationship of a cloned DNA sequence on the short arm of the X chromosome to Duchenne muscular dystrophy

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Duchenne muscular dystrophy (DMD) is one of the most common and serious human X-linked disorders. It occurs at a frequency of up to 1 in 5,000 newborn males in most populations studied, with about one-third of all cases due to new mutations[1]. The primary biochemical defect remains unknown, and no proven prenatal diagnostic test exists, although raised serum creatine kinase levels act as a somewhat equivocal guide to carrier females[2]. Previous studies have shown no measurable genetic linkage of the *DMD* locus with any X-chromosome marker[3,4]. Therefore, if a cloned sequence of the X chromosome could be used to define the locus, and to provide a closely linked set of markers, it would be of considerable importance in the prediction and prevention of DMD, as well as a step towards identifying the basic biochemical defect causing the disease. We present here evidence of an X-chromosome sequence, defined by its restriction enzyme polymorphism, that is loosely linked to *DMD*, at a distance of approximately 10 centimorgans, as determined by studies on nine informative families. The polymorphism occurs in 29% of women in a control London population and in 22% of carriers for *DMD*. The linkage data support cytogenetic evidence that *DMD* is on the short arm of the X chromosome. The object of this letter is to encourage others to make use of our probe, which seems to be linked to the *DMD* locus.

Restriction fragment length variation results from changes in the primary DNA sequence, such as single base changes introducing or deleting a restriction site, or sequence deletions, additions or translocations affecting the length of DNA between sites. These DNA sequence polymorphisms are inherited in a mendelian fashion and may be used in conventional linkage studies[1–4]. Variations in restriction sites around the human β-globin genes have been estimated to occur once in every 100 base pairs[5]. Polymorphisms are sufficiently frequent in the human genome to assume that one will be found adjacent to any random cloned DNA probe several kilobases (kb) in length. A set of such sequences can be used to construct a linkage map of the entire human genome, or of a particular chromosomal region[6,10–13].

We have previously cloned a collection of sequences from the human X chromosome in λ phage after sorting using a cytofluorimeter; approximately 50,000 clones were obtained, sufficient to ensure that >90% of sequences from the X chromosome are represented in the library[14].

Single-copy sequences were identified in the X-chromosome library using Benton–Davis screening[15] to total labelled human DNA; clones containing repetitive elements present at >100 copies in the genome hybridize and are labelled in the reannealing conditions used[16]. One of the single-copy clones obtained, given the laboratory acronym λRC3, was characterized by

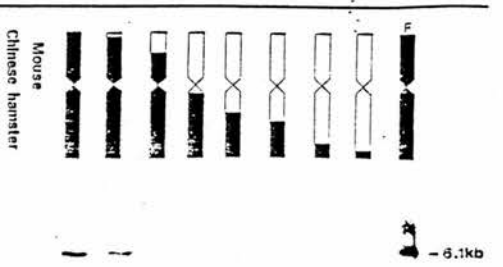

Fig. 1 Localization of λRC3 to the region Xp21–Xp223 using *Eco*RI-digested DNA from a panel of rodent–human cell hybrids. Positioned above each lane is a representation of the human X chromosome, shaded to show the portion which is present in the hybrid cell line from which the DNA was obtained (from left to right): Chinese hamster parental cell line, mouse parental cell line; hybrids containing part or all of the human X chromosome: Xpter–Xqter, Xp223–Xqter, Xp21–Xqter, Xq12–Xqter, Xq21–Xqter, Xq22–Xqter, Xq26–Xqter, Xq28–Xqter, fibroblast cell line (F). These cell lines were characterized cytogenetically and by isozyme markers in the department of Genetics, University of Freiburg. High molecular weight total DNA was extracted, digested and analysed by Southern blotting as previously described[23,24]. Blots were washed in 1×SSC at 65 °C and autoradiographied at −70 °C for 72 h.

hybridization to *Eco*RI-digested human DNA. DNA from various rodent–human hybrid cell lines containing X chromosomes or fragments as the only human genome component, and mouse DNA using Southern blots[17].

This clone contains a 6.1-kb insert of human DNA and hybridizes to a single 6.1-kb band in *Eco*RI-digested human genomic DNA, and DNA from HORL9.X cells (a mouse–human hybrid cell line in which the only human chromosome is the X chromosome), but not to mouse DNA (Fig. 1). It hybridizes to 46XY, 46XX and 48XXXX genomic DNAs with an intensity relative to the number of X chromosomes present (not shown), demonstrating that it is X-chromosome specific. In hybridizations to total human DNA it gives a slight background after moderate stringency washes (1×SSC) which disappears after washing filters at a higher stringency (0.1×SSC) and does not interfere with the analysis reported here.

Somatic cell hybrids were used to locate the DNA sequence cloned in λRC3 to a region of the X chromosome. Hybridization of the clone to DNA samples from various rodent–human cell lines containing portions of the human X chromosome localized λRC3 to the region Xp21–Xp223 (Fig. 1). There was no correlation between hybridization and the spectrum of autosomes present in these cell lines.

Table 1 Linkage data for Duchenne muscular dystrophy, probe λRC3, Xg blood group and colour blindness (CB)

θ	DMD–λRC3 $n=9$	DMD–Xg $n=10$	DMD–CB $n=3$	λRC3–Xg $n=4$
0.01	+0.393	−13.403	−9.781	−0.711
0.05	+1.535	−6.667	−5.018	−0.099
0.10	+1.766	−3.986	−3.082	+0.090
0.15	+1.715	−2.573	−2.028	+0.050
0.20	+1.543	−1.683	−1.343	−0.161
0.25	+1.305	−1.085	−0.865	−0.144
0.30	+1.033	−0.675	−0.524	−0.115
0.35	+0.753	−0.394	−0.283	−0.078
0.40	+0.481	−0.203	−0.123	−0.046
0.45	+0.229	−0.078	−0.030	−0.017

Results are expressed as lod scores (log of the odds) for different values of recombination fraction (θ). The number of kindreds informative for each comparison is indicated (n).

0028–0836/82/440469–03$01.00/0

JOURNAL OF VIROLOGY, Dec. 1995, p. 7497–7506
0022-538X/95/$04.00+0

Vol. 69, No. 12

Human CD4+ T-Cell Repertoire of Responses to Influenza A Virus Hemagglutinin after Recent Natural Infection

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Received 9 May 1995/Accepted 16 August 1995

The human CD4+ T-cell repertoire of responses to hemagglutinin (HA) of influenza virus A/Beijing/32/92 was examined 3 to 6 months after natural infection by using a panel of 16-mer peptides overlapping by 11 residues. Short-term CD4+ T-cell lines were derived by using full-length HAs of virus A/Beijing/32/92 from 12 unrelated, major histocompatibility complex (MHC) class I and II haplotyped adults with a history of influenza in November and December 1993 and from 6 adults with no history of influenza during the preceding 4 years but who responded to HA. In contrast to recent murine studies, the human CD4+ T-cell repertoire of responses was dominated by the recognition of highly conserved epitopes. The HA2 subunit, widely regarded as nonimmunogenic, induced strong responses in every donor. This resulted in functional cross-reactivity among influenza A viruses. Our study included one pair of unrelated donors expressing identical HLA DRB1* and DQB1* alleles and two pairs of donors sharing low-resolution MHC class II types. These pairs responded to identical peptides; furthermore, clearly identifiable patterns of response were seen in donors sharing single class II haplotypes, irrespective of the presence of other alleles and exposure history. Two conserved regions which induced responses in 17 of 18 donors were identified (residues 295 to 328 and 407 to 442). Possible implications for cross-reactive T-cell vaccines are discussed.

Influenza virus type A remains a major cause of human morbidity and mortality. Humoral immunity is hindered by antigenic shift and drift of the viral surface coat proteins (2). The major surface glycoprotein hemagglutinin (HA), which consists of two subunits, HA1 and HA2, produced by enzymatic cleavage of a precursor molecule, HA0, is responsible for virus-host cell fusion. The HA1 subunit contains both highly conserved and variable regions located at five main sites on the surface of the molecule (36); these are the targets of the host neutralizing antibodies. HA2 is conserved in structure among H3 influenza A viruses and has homology with influenza type A H1 and H2 viruses. CD4+ T-cell help is required for the production of neutralizing antibody (1, 34), and HA is a major antigen for both the murine and the human CD4+ T-cell responses to influenza virus (14, 16, 20, 24).

Murine studies have demonstrated that CD4+ T cells recognize both conserved and variable regions of HA, depending on the experimental system used (reviewed in reference 37). Thomas and associates have shown, in a series of detailed experiments, that following nasal priming with live virus, strong CD4+ T-cell responses to epitopes closely related to the highly variable, antibody-neutralizing sites of HA occur (3, 4, 10, 15, 24, 32), indicating that murine T- and B-cell epitopes can overlap (4).

Very few studies of the human CD4+ T-cell response to influenza A virus HA have been undertaken, and the responses of CD4+ T cells derived from only a single donor have been mapped in detail (19, 21); even this study employed an incomplete panel of peptides derived from only the HA1 sequence of a viral strain that had not circulated for 10 years. However, the indication from this study and two other studies using T-cell clones derived from single donors and panels of influenza viruses (14) or HAs (31) is that the human response may be directed, at least in part, towards conserved regions of HA.

In order to obtain detailed information about the human CD4+ T-cell repertoire of responses after recent natural infection, we investigated the responses of CD4+ T cells from a panel of 12 unrelated major histocompatibility complex (MHC) class I- and II-typed adult donors with a history of influenza virus infection during the winter of 1993 to 1994 (recent influenza virus donors). We employed HA and peptides derived from influenza virus A/Beijing/32/92, the main H3 influenza virus circulating at the time of our recent influenza virus donors' illnesses. We aimed to examine the balance of conserved and variable epitope recognition following natural infection, to examine the CD4+ T-cell response to both the HA1 and the HA2 subunits, and to investigate the MHC genetics of peptide recognition. We decided to study the responses of CD4+ T-cell lines rather than T-cell clones because the use of clones would have severely restricted the number of donors examined, would have led to the selection of a small number of dominant epitopes that were not necessarily representative of the CD4+ T-cell repertoire, and might therefore have introduced bias in favor of conserved-epitope recognition. We employed a panel of CD4+ T-cell lines selected with full-length HA for 2 to 3 weeks and examined their proliferative responses to HA from virus A/Beijing/32/92 and HA derived from the prototype H3 subtype virus A/Aichi/68. Epitope recognition was mapped by using a panel of 118 16-mer peptides, overlapping by 11 residues, spanning the entire sequence of A/Beijing/32/92 HA.

In order to compare the results obtained within 6 months after infection with longer-term CD4+ T-cell memory, we also studied a panel of six unrelated adult volunteers who had been

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TABLE 1. Subject details[a]

Donor	Age (yr)	Sex[b]	Haplotype		Duration of illness (days)	Cell recovery[c]
			MHC class I	MHC class II		
A	35	F	A2.68, B18.44, CW7.7/8V	DRB1*0101.11, DRB3*01/02/03, DQB1*0301.05	7	7.9
B	55	F	A1, B18.27, CW1.7	DRB1*0101.11, DRB3*01/02/03, DQB1*0301.05	14	4.5
C	35	M	A2.3, B7.44, CW5.7	DRB1*0401.15, DRB4*01, DRB5*01, DQB1*0301.0601/2	10	13.3
D	24	F	A1.72, B7.37, CW6.7	DRB1*0408.15, DRB4*01, DRB5*01, DQB1*0301.0601/2	10	4.7
E	25	F	A2, B44/63, CW1.5	DRB1*0101.0401, DRB4*01, DQB1*0301.05	10	4.5
F	21	F	A2.3, B7.44, CW5.7	"DRB1*03.04, DRB3*01/02/03, DRB4*01, DQB1*02"[d]	14	4.4
G	40	F	A2, B15.65, CW3.10	DRB1*10.1302, DRB3*01/02/03, DQB1*05.0604-8	14	13.4
H	24	F	A11.24, B52.75, CWND	DRB1*0403.12, DRB3*01/02/03, DRB4*01, DQB1*0301.0302	14	8.1
I	25	M	A2, B14, CW5	DRB1*0102.07, DRB4*01, DQB1*02.05	14	10.4
J	24	M	A1,2, B8.52, CWND	DRB1*15.0301, DRB3*01/02/03, DQB1*02.0601/2	7	3.6
K	25	M	A24, B55.62, CW9	DRB1*1302, DRB3*01/02/03, DQB1*02.06	7	4.4
L	25	F	A30.68, B41.51, CW(1501-3),(41-42)	DRB1*1301.1303/4, DRB3*01/02/03, DQB1*0301.603	5	7.0
M	38	M	A2.3, B7.51, CW1.7	DRB1*0101, DQB1*05	0	1.2
N	30	M	A2.66, B27.51, CW1.7	DRB1*0103.08, DQB1*0301.04	0	0.7
O	42	M	A2.31, B51.60, CW4.10	DRB1*0101.0402, DRB4*01, DQB1*0302.05	0	0.6
P	34	M	A2.28, B7.17, CW6.7	DRB1*15.07, DRB4*01, DRB5*01, DQB1*0303.601/2	0	0.6
Q	36	F	A2.19, B14.18, CWND	DRB1*0101.12, DRB3*01/02/03, DQB1*0301.05	0	2.1
R	28	M	ND[e]	DRB1*0101.07, DRB4*01, DQB1*02.05	0	1.5

[a] Subjects A to L had a history of influenza during November and December 1993; subjects M to R had no history of influenza during the preceding 4 years.
[b] F, female; M, male.
[c] Ratio of cells recovered at day 21 in culture to number of PBMC initially seeded, calculated as follows: (number of cells recovered on day 7/number of PBMC originally seeded) × (number recovered on day 14/number seeded on day 7) × (number recovered on day 21/number seeded on day 14).
[d] Subject F was class II typed serologically only, with the typing reported in molecular typing nomenclature for comparison.
[e] ND, not done.

found to make polyclonal responses to HA but who gave no history of influenza during the preceding 4 years (control donors).

MATERIALS AND METHODS

Subject selection. Twelve unrelated healthy adult donors (ages, 21 to 55 years) with a history of influenza virus infection during November and December of 1993 were studied (recent influenza donors, subjects A to L) 3 to 6 months after infection. None had ever been vaccinated against influenza virus. A positive history was taken to be fever, headache, sore throat, myalgia, and severe lassitude lasting several days during a period when A/Beijing/32/92-like strains were circulating. Many of the subjects had extended periods of lassitude lasting several months and/or persistent cough. Three of the donors (D, G, and I) had been examined for responses to HA during the summer of 1993 and were found to be nonresponders.

An additional group of six healthy subjects (ages, 28 to 42 years), with no history of influenza-like illness during the previous 4 years but who made a strong polyclonal T-cell response to HA, were studied as a comparison (control donors, M to R). Subject details are summarized in Table 1.

MHC typing. MHC class I typing was performed by a combination of serological typing at the Tissue Typing Laboratory, St. Mary's Hospital, London, United Kingdom (33), and molecular typing using sequence-specific primers (PCR-SSP) at the Tissue Typing Laboratory, Churchill Hospital, Oxford, United Kingdom (9, 30). MHC class II typing was performed by molecular typing using PCR-SSP at the Tissue Typing Laboratory, Churchill Hospital (26), with the exception of one donor (F) who was typed serologically at St. Mary's Hospital. All donors' tissue types are presented in the format recommended in "Nomenclature for Factors of the HLA System, 1994" (6).

Antigens. X117 recombinant A/Beijing/32/92 HA was the generous gift of R. Brands, Solvay Duphar, Weesp, The Netherlands. The preparation was egg derived and contained the entire HA1 and HA2 subunits. The preparation was contaminated with a trace of nucleoprotein. HA from A/Aichi/68 was the generous gift of A. Hay and J. Skehel, National Institute for Medical Research, Mill Hill, London, United Kingdom, and was obtained by bromelain cleavage (7). A series of overlapping peptides were derived from the nucleotide sequence of the HA1 subunit of A/Beijing/32/92 and the HA2 subunit of the closely related virus A/Hong Kong/90, as sequence information for the HA2 subunit of A/Beijing/32/92 was unavailable at the time of peptide synthesis. Unpublished sequence information for both viruses was kindly supplied by N. Cox (Centers for Disease Control and Prevention, Atlanta, Ga.). Peptides were synthesized by using F-moc chemistry on a Multipeptide Synthesis block BT7400 (Cambridge Research Biochemicals, Wirral, United Kingdom) with Pepsyn KB resins by the method of Fairchild et al. (13). In brief, couplings were performed by using F-moc side chain protected pentafluorophenyl or oxobenzotriazine amino acid esters in the presence of 1-hydroxybenzotriazole. Full-length peptides were deprotected with trifluoroacetic acid-phenol-thioanisole-ethanedithiol in the ratio of 94:2:2:2 and cleaved with 0.4 M NaOH. The resulting solutions were neutralized with HCl to yield peptides in saline solution. The peptides were 16 residues long, overlapping normally by 11 amino acids. The composite primary amino acid sequence used to synthesize the peptides and individual peptide sequences are shown in Fig. 1, which also shows the residue changes between the HAs from A/Aichi/68 and A/Beijing/32/92 as an indication of the variable regions of H3 HA. The numbering of peptides used in this study is based on the precursor molecule HA0.

Establishment of CD4+ T-cell lines. Venous blood was collected between 3 and 6 months after infection, and peripheral blood mononuclear cells (PBMC) were separated by Ficoll-Hypaque density centrifugation. A total of 25×10^6 PBMC were cultured for 7 days in 24-well tissue culture plates at 37°C with 5% CO_2 with HA from A/Beijing/32/92 (0.1 μg/ml) at a density of 5×10^6 cells per well, in 2 ml of complete medium (RPMI 1640 supplemented with 2 mM L-glutamine, 100 IU of penicillin-streptomycin [Gibco, Life Technologies, Paisley, Scotland] per ml, and 5% screened, inactivated human AB+ serum [Sigma, Poole, Dorset, England]). The remaining PBMC were stored in liquid N_2 and subsequently used as antigen-presenting cells (APCs).

At day 7, 4×10^6 T cells were used to analyze the response to HA and peptides as described below and the remaining T cells were restimulated, at a density of 1.0×10^6 per well, with equal numbers of irradiated (3,000 rads) autologous PBMC preincubated at 37°C in 5% CO_2 in complete medium for 1 h with HA from A/Beijing/32/92 (0.01 to 0.1 μg/ml). At 24 and 72 h, the lines were supplemented with a source of interleukin 2 (Lymphocult T; Biotest Folex, Frankfurt, Germany) (10%, vol/vol). The T-cell lines were maintained in culture for 3 to 4 weeks as described above, with the addition of fresh, irradiated autologous prepulsed PBMC every 7 days.

Analysis of T-cell specificities by using overlapping peptides. Autologous, irradiated (3,000 rads) PBMC (4×10^4/well) were incubated with whole antigen (HA from A/Beijing/32/92 [0.01 to 0.1 μg/ml] or HA from A/Aichi/68 [1.0 μg/ml]) or peptide pools (five peptides each at a concentration of 10 μg/ml) for 1 h at 37°C in 5% CO_2 in complete medium and subsequently cultured with 4×10^4 responder T cells per well in round-bottomed 96-well plates. At 48 h, the cells were pulsed with [^{3}H]thymidine (1 μCi per well; Amersham International PLC, Amersham, United Kingdom) and harvested 16 h later. Proliferation, as correlated with [^{3}H]thymidine incorporation, was measured by liquid scintillation spectroscopy. Responses of at least five times the geometric mean of the background (T cells, autologous irradiated PBMC, and medium) were scored as positive. Mapping with individual peptides (10 μg/ml) was conducted at day 21 or 28 as described above.

MHC class II restriction studies. Monoclonal anti-HLA-DR (L243) (22), anti-HLA-DP (B7/21) (35), and anti-HLA-DQ (SPV-L3) (a gift from H. Spitz, DNAX, Palo Alto, Calif.) antibodies were isolated from hybridoma culture

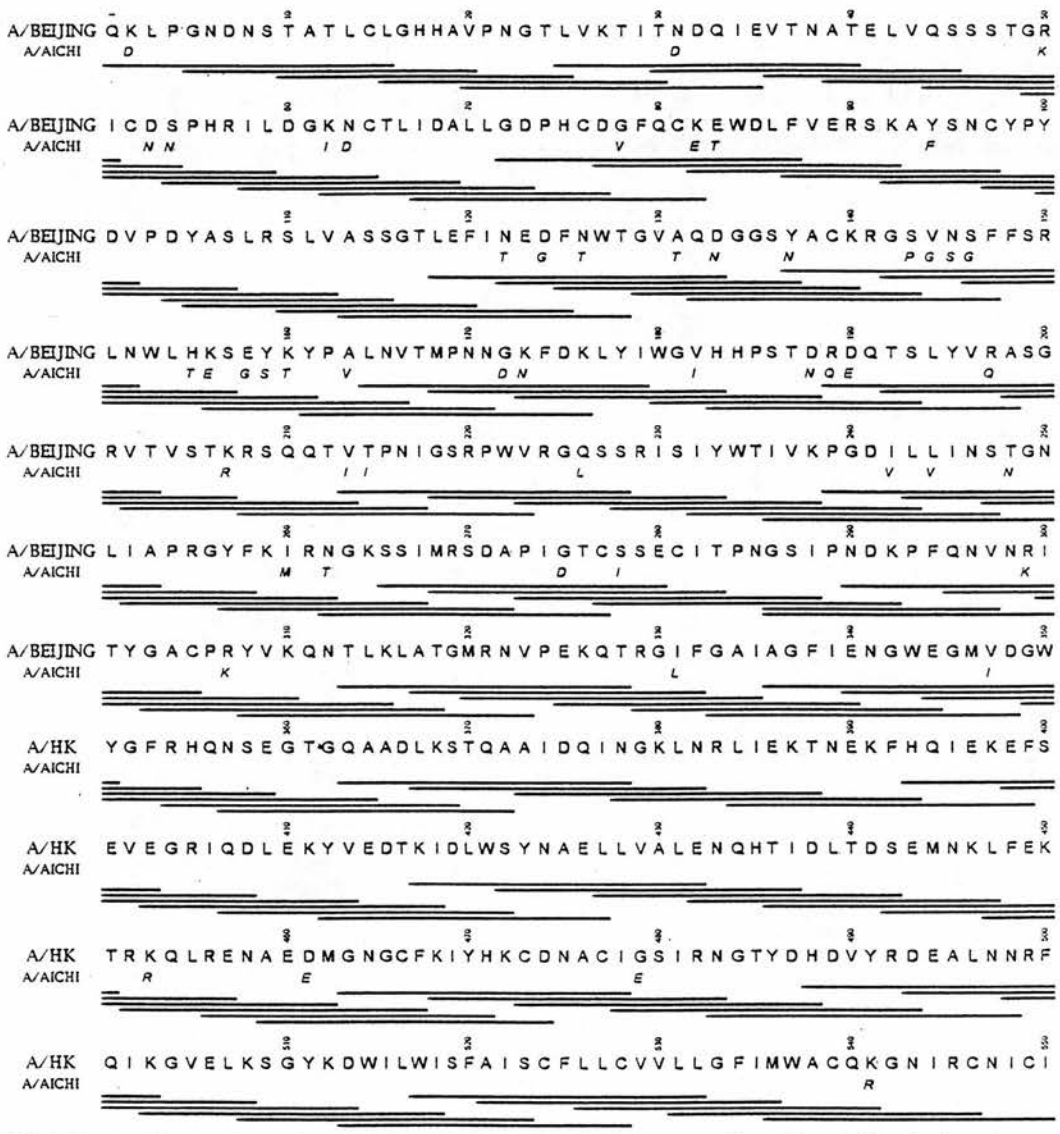

FIG. 1. Sequences of HA from A/Beijing/32/92 and peptides used in this study. The amino acid sequence used for peptide synthesis, based on the putative sequence of HA0, is shown. The HA1 subunit (residues 1 to 328) is based on A/Beijing/32/92. The HA2 subunit (residues 330 to 550) is based on the closely related A/Hong Kong/90, as no sequence information on the HA2 subunit of A/Beijing/32/92 was available at the time of synthesis. Sequence differences between A/Beijing/32/92 and A/Aichi/68 HAs (in italics below main sequence) are indicated. A/Beijing, A/Beijing/32/92 HA; A/Aichi, A/Aichi/68 HA; A/HK, A/HongKong/90 HA.

supernatants and purified with protein A-Sepharose. Autologous irradiated (3,000 rads) PBMC (4×10^4/well) were incubated for 30 min with 10 μg of antibody per ml before the addition of antigen (1 and 5 μg of individual peptides per ml). After 1 h of incubation at 37°C in round-bottomed 96-well plates in complete medium, T cells (4×10^4/well) were added. The cells were cultured, pulsed, and harvested as described above.

Cross-reactivity of T-cell lines among a panel of influenza A viruses. The following freeze-dried viruses were kind gifts of P. Chakraverty, Public Health Laboratory Service, Colindale, London, United Kingdom: H1N1, A/Formosa/1/47, A/Taiwan/1/86, and A/Fiji/2/88; H2N2, A/Singapore/1/57; and H3N2, A/Victoria/3/75, A/Bangkok/1/79, and A/England/427/88. Between 1 and 10 hemagglutinating units of virus was preincubated with autologous irradiated APCs for 1 h, which were then cultured with T cells as described above.

Fluorescence flow cytometry. T-cell lines were stained directly with saturating concentrations of fluorescein isothiocyanate-conjugated murine monoclonal antibodies anti-Leu-4 (CD3), anti-Leu-3a (CD4), and anti-Leu-2a (CD8) (Becton

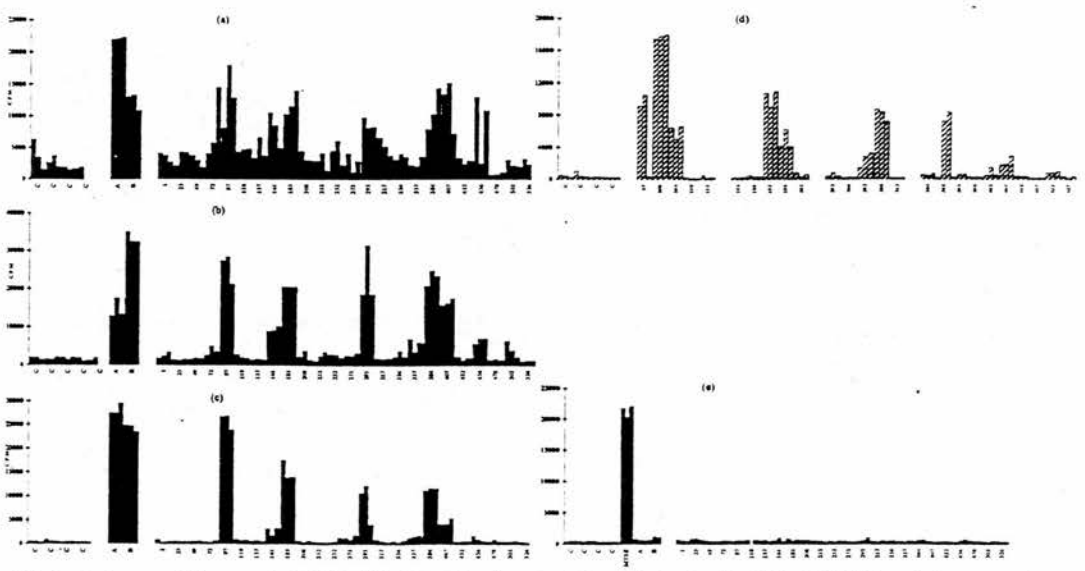

FIG. 2. Development of HA response by CD4⁻ T-cell line derived from donor A and selected for 3 weeks with A/Beijing/32/92 HA (a to d), plus response of control MTSE-specific CD4⁻ T-cell lines to HA and peptides (e). T-cell proliferation in response to A/Aichi/68 HA, A/Beijing/32/92 HA and peptide pools was tested at 7 (a), 14 (b), and 21 (c) days by using irradiated autologous prepulsed PBMC as APCs. At 48 h, the T-cell lines were pulsed with [^{3}H]thymidine, and they were harvested 16 h later. The y axis represents individual data for each well of a triplicate: C, control (proliferative response of T-cell line to complete medium and autologous APCs); A, response of T-cell line to A/Aichi/68 HA (1.0 μg/ml); B, response to A/Beijing/32/92 HA (0.1 μg/ml). Peptide pools are labelled according to the number of the first residue of the first peptide in the pool (five peptides per pool, 10 μg of each peptide per ml). The y axis represents counts per minute. (d) More-detailed mapping with single peptides (10 μg/μl) conducted at day 28. (e) Response of MTSE-specific T-cell line to HA and peptides used in this study. The CD4⁻ T-cell line was derived from a donor nonresponsive to A/Beijing/32/92 HA by stimulating PBMC with 5 μg of MTSE per ml. Bar MTSE, response to 5 μg of MTSE per ml.

Dickinson Immunocytometry Systems, San Jose, Calif.). Viable cells, identified by their ability to exclude propidium iodide, were analyzed by flow cytometry with an Epics Profile II (Coulter Electronics, Luton, Beds, United Kingdom). The cell population was analyzed by gating on the volume and light scatter characteristics.

RESULTS

Subjects. The panel of donors with a history of influenza during November and December 1993 (recent influenza virus donors) had a wide range of MHC class II haplotypes (Table 1). One pair, donors A and B, shared MHC HLA DRB1* and DQB1* haplotypes (DRB1*0101,11, DRB3*01/02/03, and DQB1* 0301,05). A second pair, donors C and D, had identical low-resolution MHC class II types (DRB1*04,15, DRB4*01, DRB5*01, and DQB1*0301,0601/2) and differed only in HLA DRB1*04 subtype. Three donors (D, G, and I) did not proliferate in response to HA from A/Aichi/68 during the summer of 1993 but recognized both A/Beijing/32/92 and A/Aichi/68 HAs after influenza-like illness in November and December 1993 (not illustrated).

The second panel of donors, with no history of influenza during the preceding 4 years (control donors), also had a range of MHC class II haplotypes, although four shared the DRB1* 0101 allele (Table 1). Donor O shared low-resolution HLA DR and DQ types with donor E, who had a history of recent influenza.

In total, of the 18 donors in the two groups, 7 shared the DRB1*0101 DQB1*05 haplotype, 5 shared the low-resolution HLA DRB1*04 type (but had several different subtypes), 4 shared the DRB1*15 haplotype, 3 shared the DRB1*13 haplotype, 2 shared the DRB1*07 haplotype, and 2 shared the DRB1*11 haplotype. In addition, the responses of donors with HLA DRB1*0102, -0103, -03, -08, -10, and -12 haplotypes were examined.

Lack of mitogenicity of HAs and peptides. The antigens used in this study were tested for mitogenicity by using a human CD4⁻ T-cell line specific for *Mycobacterium tuberculosis* soluble extract (MTSE). No mitogenicity (greater than three times the geometric mean) was induced by A/Beijing/32/92 or A/Aichi/68 HA or any of the synthetic peptides (Fig. 2e).

Characteristics of T-cell lines. T-cell lines derived from the recent influenza virus donors and selected with A/Beijing/32/92 HA expanded at least fourfold in culture over 3 weeks, in contrast to those derived from the control donors, which, with the exception of donor Q's T-cell line, showed no significant expansion (data in Table 1).

Analysis of two T-cell lines by flow cytometry showed that the ratio of CD4⁻ cells to CD8⁻ cells progressively increased from days 7 to 21. Six T-cell lines tested at 21 days were >90% CD3⁻, 78 to 88% CD4⁻, and 2 to 10% CD8⁻ (not illustrated).

Examples of the proliferative responses obtained from donor A at 7, 14, and 21 days in culture are shown in Fig. 2 (a to c). The dominant responses are to peptide pools 97-128, 183-217, 295-328, 384–418, and 407–442, while the background proliferation progressively drops with time. In general, the patterns of response to major epitopes remained stable, though there was some loss of response to minor epitopes. Figure 2d shows the results of more-detailed analysis of epitope recognition by this line using single peptides at 28 days. Because of the increased ratio of CD4⁻ T cells and low background pro-

liferation, we chose to analyze peptide specificity at 21 days whenever possible.

Responses of CD4⁺ T-cell lines derived from unrelated donors with a history of recent influenza to HA and peptides. The recent influenza virus donors responded to both A/Beijing/32/92 and A/Aichi/68 HAs (Fig. 3). Marked differences in patterns of peptide recognition were seen between individual T-cell lines from donors differing in MHC class II haplotype. Without exception, every CD4⁺ T-cell line mounted a significant proliferative response to the HA2 subunit.

When results for individual recent influenza virus donors during the second and third weeks of culture were combined, we found that almost the entire protein sequence as represented by the peptide pools could induce proliferative responses. Three regions of HA, represented by peptide pools 97-128, 295-328, and 407-442, were recognized by 10 of 12, 12 of 12, and 11 of 12 donors, respectively, during the second and/or third weeks. A further three regions (pools 183-217, 357-393, and 384-418) were recognized by 8 of 12 donors. Studies with single peptides within the dominant regions showed that the responses usually localized to identical individual peptides or a pair of overlapping peptides, representing regions of HA that have been structurally conserved over many years among H3 influenza A viruses (not illustrated). Pool 407-442 was found to contain at least two immunogenic regions.

Responses of CD4⁺ T-cell lines from adult donors with no history of influenza during the preceding 4 years to HA and peptides. The responses of the six control donors (M to R) were, in general, to regions of HA similar to those of the recent influenza virus donors, though not as strong (Fig. 4). Once again, every T-cell line responded to the HA2 subunit. Pools 97-128, 295-328, and 407-442 were recognized by four, five, and six of six donors, respectively.

Influence of MHC class II on T-cell-recognition of HA peptides. It is striking that the responses of donors A and B (sharing DRB1*0101,11, DRB3*01/02/03, and DQB1*0501,0301) to the peptide pools were very similar; both responded to pools 97-128, 183-217, 295-328, and 407-442, and studies with single peptides demonstrated that the responses were localized to identical peptides or pairs of overlapping peptides within the pools (not illustrated). Donors C and D (DRB1*04,15, DRB4*01, DRB5*01, DQB1*0301,0601/2) differed in DRB1*04 subtype (0401 and 0408, respectively), but both T-cell lines responded strongly to pools 1-25, 25-59, 97-128, 273-305, 295-328, 407-442, and 456-488. An additional response to 252-283 was made by donor D, and again, when single-peptide studies were undertaken, dominant responses were found to be directed to identical individual peptides. Donors E and O, who differed in history of exposure to influenza virus but shared low-resolution HLA DRB1*01,04, DRB4*01, and DQB1*03,05 types, made strong responses to identical peptide pools, though there were differences in the relative strengths of their responses.

Similarities in patterns of response were also seen between individuals who shared a single MHC class II haplotype. This was particularly evident for HLA DRB1*0101 for subject E, who shared DRB1*0101 and DQB1*0301,05 with subjects A and B and responded to pools 97-128, 183-217, 295-328, 384-418, and 407-442. The responses of cell lines from subjects M, O, and R, who had no history of recent influenza and who shared the MHC class II alleles DRB1*0101 and DQB1*05, were very similar to those of subjects A, B, and E, with all six lines responding strongly to peptide pools 97-128, 295-328, and 407-442. Subject Q (DRB1*0101,12, DRB3*01/02/03, DQB1*0301,05) responded to pools 295-328 and 407-442 but not to pool 97-128.

In contrast, marked differences were seen in response patterns of donors with different subtypes of HLA DRB1*01: neither donor I (DRB1*0102) nor donor N (DRB1*0103) followed the pattern seen for the DRB1*0101 donors.

Results of studies with anti-MHC class II antibodies. The results of the MHC restriction studies using antibodies to MHC class II on two T-cell lines are shown in Fig. 5. The majority of responses by both donors were inhibited by anti-HLA-DR antibody and were therefore HLA DRB1* restricted. Interestingly, donor D showed some increase in proliferative response to peptide pairs 20/25, 97/100, and 303/308 following preincubation with anti-HLA-DP antibodies.

Influenza virus cross-reactivity. Six T-cell lines from the recent influenza virus donors, including all three donors (D, G, and I) who were nonresponsive to A/Aichi/68 HA prior to November or December 1993, were examined for cross-reactivity among a panel of H1, H2, and H3 influenza A viruses (Fig. 6). All were cross-reactive, although the relative strengths of responses to H1, H2, and H3 viruses varied considerably. T-cell line I showed a stronger response to H3 viruses than to the H1 or H2 viruses. This T-cell line was dominated by responses to HA2. Studies with single peptides localized the response to regions 384 to 399, 403 to 422, 427 to 442, and 463 to 483 (not illustrated). In three of these four regions, radical changes (residue 388, T to M; 404, G to K and R; 412, Y to K; 417, F to K; 430, A to L; and 440, T to H) which probably account for these observed differences in response occur between H3 and H1 and H2 influenza A viruses.

DISCUSSION

We report the first major study of the human CD4⁺ T-cell repertoire of responses to HA after recent natural infection. CD4⁺ T-cell lines were derived from a panel of 12 unrelated MHC class I- and II-haplotyped adult donors with a history of influenza during November and December 1993 (recent influenza virus donors) and a panel of 6 adult donors who responded to HA but who had no history of influenza in the past 4 years (control donors). CD4⁺ T-cell lines were selected by using full-length HA from A/Beijing/32/92 and epitope recognition mapped by using a panel of 16-mer peptides, overlapping by 11 residues, covering the entire sequence of HA1 and HA2. Cross-reactivity was investigated by using the original 1968 H3 influenza A virus HA and a panel of influenza A viruses. Results have been interpreted in the context of the donors' MHC class II haplotypes.

HA was highly immunogenic in all cases. We expected MHC class II to play an important role in HA epitope selection in association with an individual's exposure history and other factors. It is remarkable, however, that proliferative responses by the short-term T-cell lines from unrelated donors A and B, who are both DRB1*0101,11, DRB3*01/02/03, DQB1*0301,05, were both directed predominantly to peptide pools 97, 183, 295, 384, and 407, with only some quantitative differences. Analysis using single peptides revealed that their responses were to identical peptides or pairs of overlapping peptides (not illustrated). Donors E and O, who share low-resolution HLA DR and DQ types but differ in exposure history, also recognized identical peptide pools. This similarity in pattern of response was not limited to donors with such close MHC class II matches, as all seven donors who expressed DRB1*0101 and DQB1*05 alleles, irrespective of differences in their history of exposure to influenza virus and differences in other HLA class

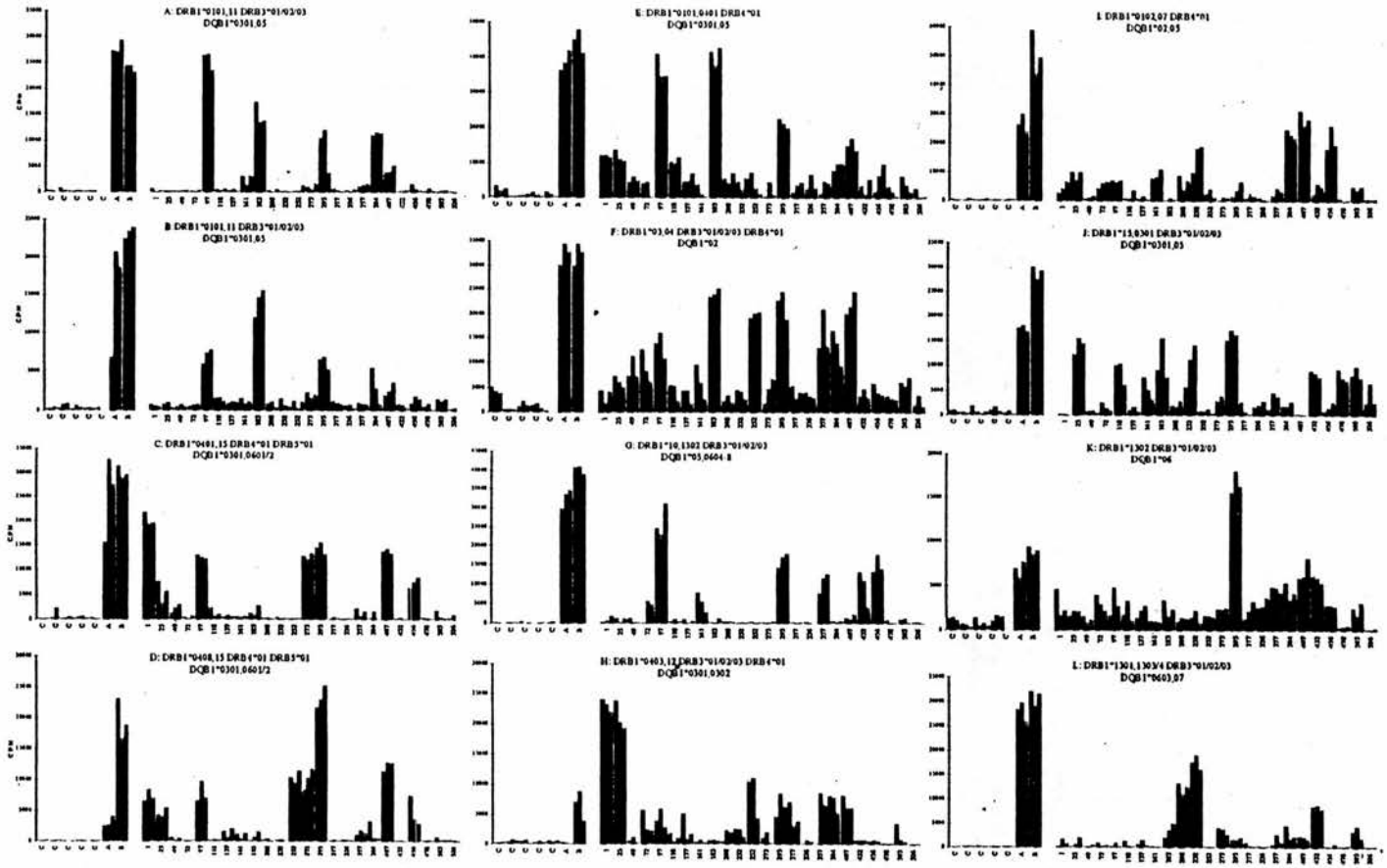

FIG. 3. HA response by CD4+ T-cell lines from 12 unrelated donors (A to L) with a history of recent influenza and selected for 2 or 3 weeks with A/Beijing/32/92 HA. A/Beijing/32/92 HA, A/Aichi/68 HA, and peptide pools were presented to T-cell lines as described for Fig. 2. The class II MHC types of the donors are shown.

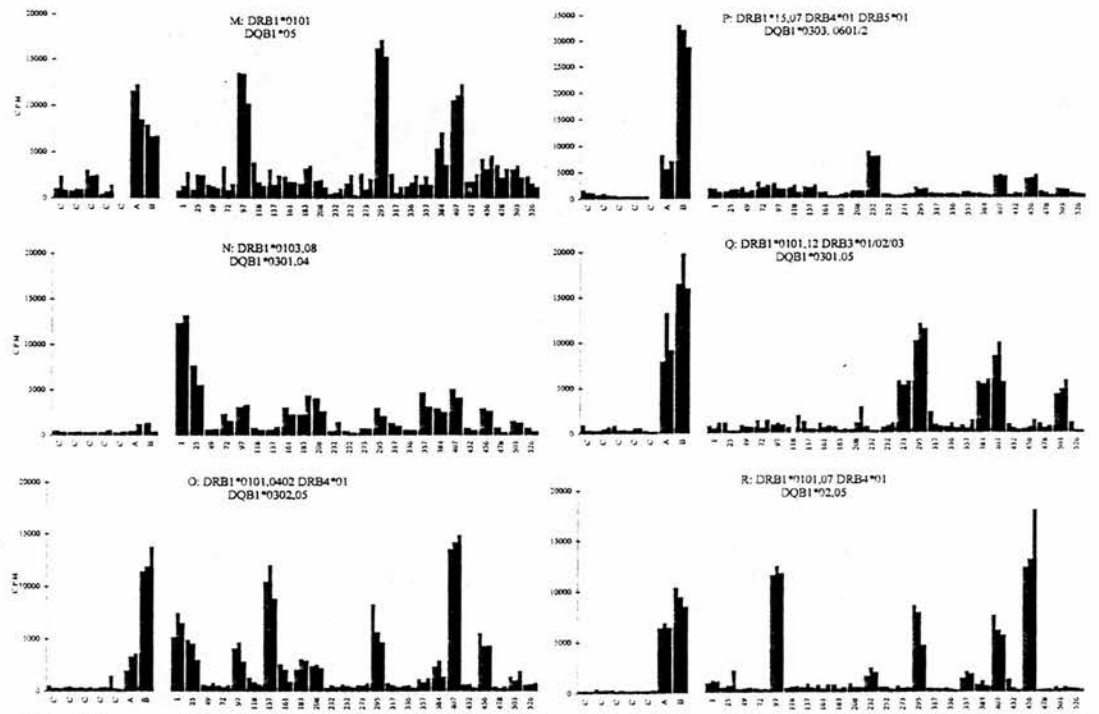

FIG. 4. HA responses of CD4⁻ T-cell lines from donors with no history of influenza during the last 4 years. For details, see the legend to Fig. 2. Three donors had previously been frequently exposed to influenza virus, donors N and P, who were practicing physicians, and donor O, who had worked extensively with H3 influenza A viruses 10 years previously.

II alleles, showed similar patterns of T-cell recognition (except for donor Q, who failed to respond to pool 97-128).

As predicted by peptide binding studies (11, 12), the subjects who expressed other subtypes of HLA DR1, donors I (DRB1*0102) and N (DRB1*0103), in peptide recognition differed from both the DRB1*0101 donors and each other. There are only limited structural differences between these alleles (changes of V to A at residue 85 and of G to V at residue 86 for DRB1*0102 and changes of L to I at residue 67, Q to D at residue 70, and R to E at residue 71 for DRB1*0103). Six donors shared the low-resolution HLA DRB1*04 haplotype; two of them, donors C (DRB1*0401) and D (DRB1*0408), also shared DRB1*15 and had very similar patterns of peptide responsiveness. The variations between these donors may reflect the slight difference between DRB1*0401 and DRB1*0408 (change at position 71 from K to R). Identifiable patterns of response are also evident for HLA DRB1*07, with donors P, I, and R all responding strongly to pool 456-488.

Most interestingly, HA2 (residues 330 to 550), which has been regarded as largely nonimmunogenic (5), induced strong responses in every donor. Furthermore, with the exception of the N and C termini of HA2, every peptide pool representing HA2 induced a response in at least 3 of 12 recent influenza virus donors. One conserved region (residues 407 to 442) induced responses in 11 of 12 recent influenza virus donors during the second or third week in culture; this region is unchanged among H3 influenza A viruses and has homology with H1 and H2 viruses. Two additional highly conserved regions (residues 357 to 393 and 384 to 418) were recognized by 8 of 12 recent influenza virus donors. We are aware of only one preliminary study of the human CD4⁻ T-cell response to HA2: unselected PBMC were derived from 14 donors, who were not tissue typed, and limiting dilution analysis showed T-cell responses to at least three regions of HA2 (29). One murine study has demonstrated cross-reactive CD4⁻ T-cell recognition of HA2 after nasal infection with live virus (though no epitopes were determined) (18). A second murine study demonstrated responses to HA2 following vaccination with HA2 alone and characterized two epitopes (residues 425 to 437 and 499 to 511) (17).

Regarding the HA1 subunit (residues 1 to 328), two regions, residues 97 to 128 and 295 to 328, were the most frequently recognized, inducing responses in 10 of 12 and 12 of 12 recent influenza virus donors, respectively. Studies with single peptides localized the majority of responses to residues 100 to 115 and 306 to 323, which are conserved among H3 influenza A viruses (not illustrated). The second region has sequence homology with H1 and H2 viruses and is closely related to the dominant epitope originally described by Lamb et al. (who used a different panel of peptides) (19, 21). One peptide pool which contained a variable region within the H3 influenza A viruses, pool 183-217, induced responses in 8 of 12 donors. The majority of responses localized within this region were to residues 192 to 212, which have been relatively conserved during

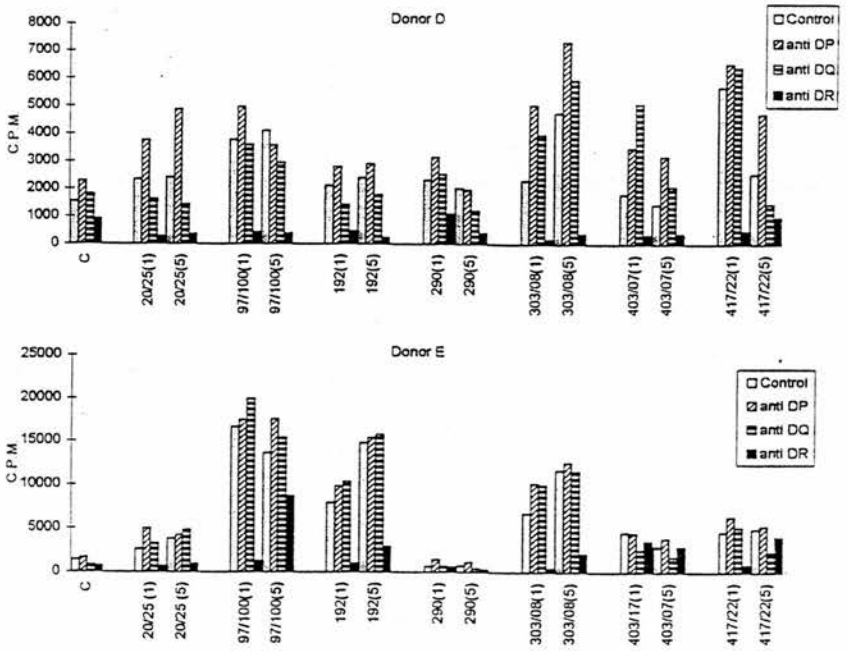

FIG. 5. Effect of MHC class II blocking antibodies on the response to dominant peptides. Autologous PBMC were incubated with 10 μg of anti-HLA-DP, anti-HLA-DQ, or anti-HLA-DR antibodies per ml or with medium (control). Individual peptides or pairs of peptides (1 and 5 μg/ml) were added to the PBMC and incubated at 37°C for 1 h prior to addition of responder $CD4^+$ T cells. Further experimental details are described for Fig. 2. Numbers on the *x* axis refer to the individual peptides or pairs of peptides; numbers in parentheses refer to the dose of peptide (in micrograms per milliliter).

the past decade (changes: residue 193, S to N; 197, Q to R; 201, R to K; and 207, K to R). Responses of $CD4^+$ T-cell lines derived from control donors M to R were examined as a comparison of results after 3 to 6 months and longer-term $CD4^+$ T-cell memory. The two groups recognized similar peptide pools, though the responses of the control panel were generally weaker. Interestingly, the control lines demonstrated an expansion in vitro which was severalfold lower than those of the recent influenza virus donors (Table 1). The most likely explanation is a lower T-cell precursor frequency, implying a decline in circulating $CD4^+$ T-cell memory after infection. A similar decline in circulating influenza virus-specific $CD8^+$ T-cell memory has been reported (23), and both findings are in agreement with early epidemiological surveys which showed protection from influenza virus following natural infection declining after 4 years (28).

It is interesting that viral infection induces recognition of several conserved HA epitopes by a large proportion of the population. Our findings concur with the original human $CD4^+$ T-cell studies (which were limited to single donors) in which, with the exception of one clone specific for a variable region of an H2 influenza A virus (8), the responses were either cross-reactive between H3 influenza A viruses (and sometimes H1 and H2 viruses) (14, 20) or localized to conserved regions of the HA1 subunit (21). This contrasts with recent murine studies of live-virus infection which have demonstrated strong $CD4^+$ T-cell responses to epitopes closely related to the antibody-neutralizing sites (3, 4, 10, 15, 24, 32).

Several explanations for these apparent species differences are possible. First, our donors may have been infected with viral strains differing from A/Beijing/32/92. However, the majority of influenza A virus strains isolated in London in November and December 1993 were similar to A/Beijing/32/92: four conservative substitutions (residue 75, H to N; 145, N to K; 201, R to K; and 208, R to K), three intermediate substitutions (residue 189, R to S; 214, T to I; and 276, T to N), and three nonconservative substitutions (residue 157, S to L; 219, S to F; and 226, Q to L) were recorded (12a). These changes offer an explanation for the low frequency of response to pools 137-171 and 208-243 which was seen but do not account for the dominance of $CD4^+$ T cells recognizing HA regions conserved within the H3 subtype. Alternatively, repeated exposure to influenza A viruses in humans may bias the $CD4^+$ T-cell response towards recognition of conserved HA epitopes. There is evidence for this phenomenon in the human B-cell response to HA (27).

We were fortunate that three donors (D, G, and I) failed to mount detectable proliferative responses to A/Aichi/68 HA during the summer of 1993. Following influenza-like illness in November and December 1993, all three donors made strong responses to both A/Aichi/68 and A/Beijing/32/92 HAs, demonstrating the induction of significant cross-reactivity.

Finally, differences in antigen presentation between I-A, which is equivalent to human HLA DQ, and HLA DR (equivalent to murine I-E) may account for these findings. Interest-

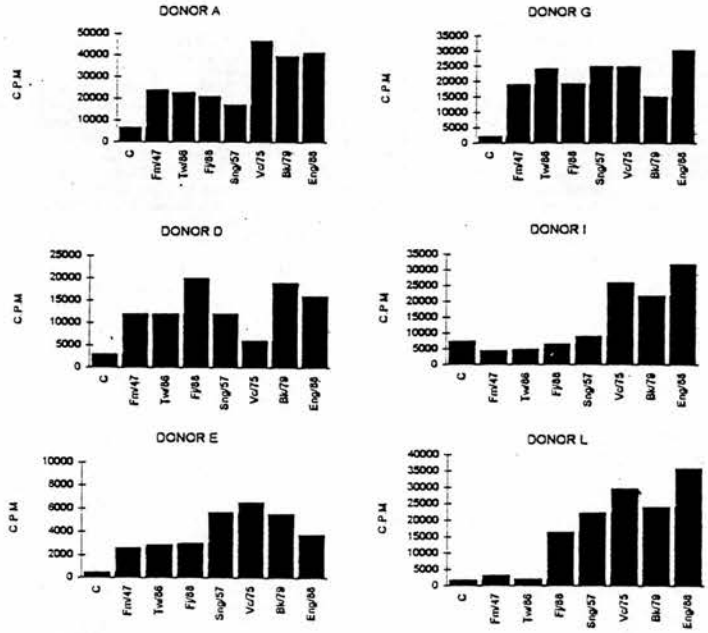

FIG. 6. Responses of six CD4+ T-cell lines to a panel of influenza A viruses. Autologous irradiated PBMC were prepulsed for 1 h with 1 to 10 hemagglutinating units of virus prior to the addition of 3-week CD4+ T-cell lines selected by A/Beijing/32/92 HA. C, control (T cells plus APCs). Viruses: Fm/47, A/Formosa/1/47; Tw/86, A/Taiwan/1/86; Fj/88, A/Fiji/2/88 (all H1N1); Sng/57, A/Singapore/1/57 (H2N2); Vc/75, A/Victoria/3/75; Bk/79, A/Bangkok/1/79; and Eng/88, A/England/427/88 (all H3N2).

ingly, Burt and coworkers have reported I-E-restricted responses directed towards more-conserved regions of HA1 (10).

Influenza virus vaccination achieves a reduction of approximately 50% in hospital admissions due to influenza and pneumonia among the elderly during an epidemic (25). Because of the persistence of antigenic variation in HA, complete protection is strain specific and generally short-lived. The partial protection obtained from vaccination may be due in part to the induction of a cross-reactive CD4+ T-cell response. This is supported by our preliminary observations of the human CD4+ T-cell response to HA following vaccination. As a few conserved regions of HA were recognized by a large proportion of our donors, it may be possible to design specific CD4+ T-cell vaccines to boost cross-reactive protection.

ACKNOWLEDGMENTS

This work was supported by a grant from the Wellcome Trust. C.M.G. is the recipient of a Wellcome Trust Advanced Training Fellowship.

We thank the following for their contributions to this study: R. Brands and Solvay Duphar for the gift of A/Beijing/32/92 HA, A. Hay and J. Skehel for the gift of A/Aichi/68 HA, P. Chakraverty for the panel of influenza viruses, N. Cox for unpublished sequence information for A/Beijing/32/92 HA, J. Ellis for information on sequence variation among influenza viruses circulating in London during November and December 1993, Mieke Van Dam for serological MHC class I typing, Martin Barnardo for assistance with MHC class II typing, and D. Wraith for helpful discussion and advice on peptide synthesis.

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JOURNAL OF VIROLOGY, July 1996, p. 4787–4790
0022-538X/96/$04.00+0

Vol. 70, No. 7

Human CD4$^+$ T-Cell Recognition of Influenza A Virus Hemagglutinin after Subunit Vaccination

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Received 30 November 1995/Accepted 16 April 1996

We have examined human CD4$^+$ T-cell recognition of influenza A/Beijing/32/92 (H3N2) virus hemagglutinin following influenza virus HANA subunit vaccination. CD4$^+$ T-cell repertoires were dominated by recognition of epitopes located in conserved regions of the molecule, in a major histocompatibility complex class II haplotype-dependent manner, analogous to that observed following natural infection.

Influenza A virus remains a major cause of human morbidity and mortality. Influvac, an influenza virus HANA subunit vaccine, is widely used for immunoprophylaxis. It is safe and induces satisfactory levels of neutralizing antibodies (11). However, because of continual antigenic variation in virus coat glycoproteins (4, 13, 14), protection by neutralizing antibodies is generally short lived. Subunit influenza virus vaccines are poor inducers of human CD8$^+$ T-cell responses (10), and until now there has never been a systematic investigation of human CD4$^+$ T-cell responses to influenza virus following any form of influenza virus vaccination. CD4$^+$ T cells play a crucial role in the control of influenza virus infection, as the production of neutralizing antibody, immunoglobulin class switching, and affinity maturation are all CD4$^+$ T-cell dependent (1, 6, 12). In addition, CD4$^+$ T cells amplify CD8$^+$ T-cell cytotoxic responses (3, 5, 9) and may participate in viral clearance more directly by the secretion of gamma interferon (2).

We recently demonstrated that in the polymorphic human population, adult CD4$^+$ T-cell repertoires to hemagglutinin (HA) at 3 to 6 months following natural infection with influenza virus A/Beijing/32/92 (H3N2)-like strains are dominated by the recognition of regions of the HA1 and HA2 subunits that are highly conserved among human influenza A virus H3 isolates and that this results in cross-reactive recognition of

TABLE 1. Donor details and expansion of CD4$^+$ T cell lines in vitro

Donor	Age (yr)	Sex[a]	Influenza episode in preceding 4 years[b]	Haplotypes		Cell yield[c]	
				MHC class I	MHC class II	Prevaccination	Post-vaccination
A	31	F	No	A3,23; B7,44; C4,0702	DRB1*1501,0701; DRB4*01; DRB5*01; DQB1*02,0601/2	2.6	4.4
B	34	M	No	A26,31; B38; C0701,1203	DRB1*0301/4,1302; DQB1*06	1.2	7.2
C	31	M	No	A2,66; B27,51; C1,7	DRB0103,080X; DQB1*0301,04	0.7	17.5
D	28	M	No	A2,29; B44,35; C4,1601	DRB1*0101,0701; DRB4*01; DQB1*02,05	1.7	10.7
E	33	M	No	A2,3; B65,51; C5,0802	DRB1*0101,1301; DRB3*01/02/03; DQB1*0501,0603	1.7	10.7
F	42	M	No	A2,31; B51,60; C4,10	DRB1*0101,0402; DRB4*01; DQB1*0302,05	0.6	5.5
G	55	M	—	A2,30; B44,51	DRB1*1501,0701; DRB4*01 DRB5*01; DQB1*02,0601/2	8.1	7.4
H	40	F	Yes	A2; B15,65; C3,10	DRB1*100X,1302; DRB3*01/02/03; DQB1*05,0604-8	13.4	17.4

[a] F, female; M, male.
[b] —, donor G, on the basis of our previous results, is likely to have had a recent subclinical infection (see the text). Donors B, F, and G had received influenza virus vaccines more than 5 years previously.
[c] Cell yield is the ratio of cells recovered at day 21 in culture to the number of peripheral blood mononuclear cells initially seeded: (number of cells recovered on day 7/number of peripheral blood mononuclear cells originally seeded) × (number recovered on day 14/number seeded on day 7) × (number recovered on day 21/number seeded on day 14).

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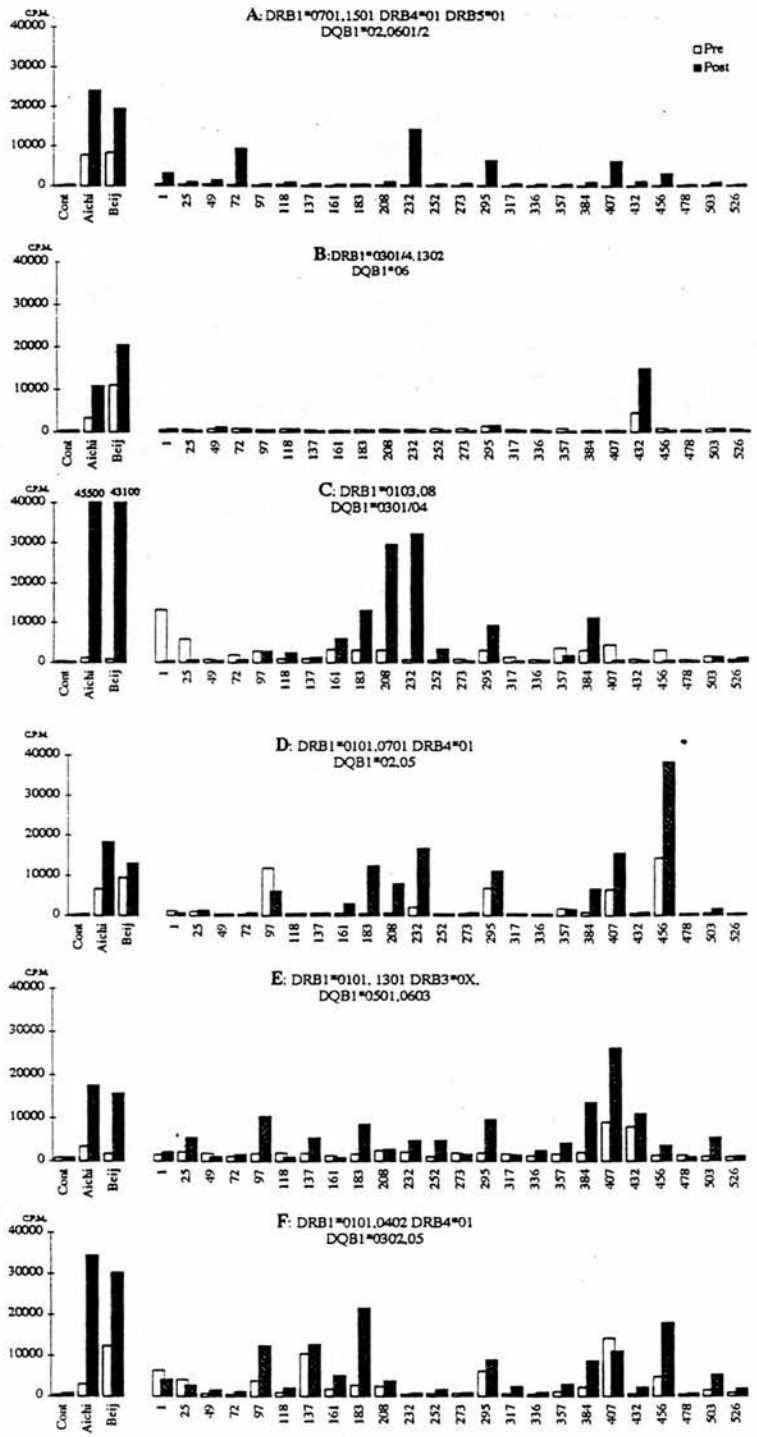
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DQB1*02,0601/2
Pre
Post
B:DRB1*0301/4,1302
DQB1*06
C: DRB1*0103,08
DQB1*0301/04
45500
43100
D: DRB1*0101,0701 DRB4*01
DQB1*02,05
E: DRB1*0101, 1301 DRB3*0X,
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F: DRB1*0101,0402 DRB4*01
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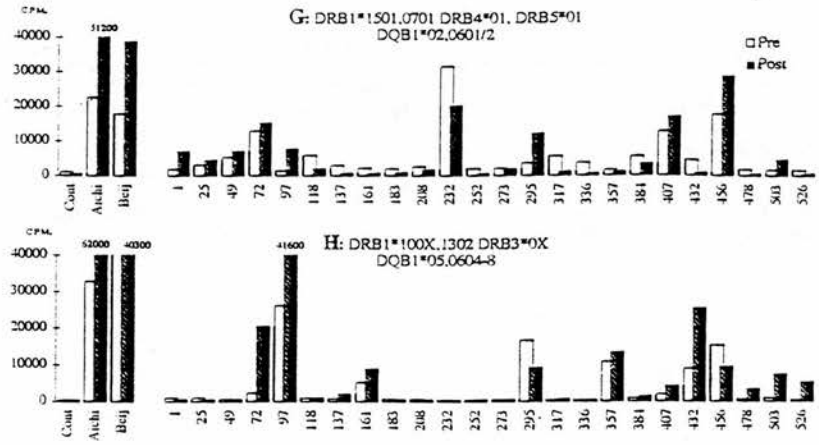

FIG. 2. HA responses by CD4⁺ T-cell lines derived from donors G and H. Donor H had a history of influenza during December 1993, and donor G, on the basis of our previous results, may have had recent subclinical influenza. The results are shown for samples obtained before (open columns) and 3 to 4 months following (striped columns) vaccination with Influvac. For details, see the legend to Fig. 1.

other human influenza A viruses (8). These findings prompted us to investigate CD4⁺ T-cell recognition of HA following virus subunit vaccination. In order to allow a direct comparison between the studies, we vaccinated eight unrelated, major histocompatibility complex (MHC)-typed, adult donors with a subunit influenza virus vaccine containing A/Beijing/32/92. HA-specific CD4⁺ T-cell lines were derived before and at 3 to 4 months following subunit vaccination. Details about the donors are given in Table 1. Donors A to G had no history of influenza for at least 5 years (though donor G may have had a recent subclinical influenza virus infection [see below]). Donor H had a history of influenza during December 1993. Subjects received 0.5 ml of Influvac, containing 15 μg each of HA from influenza A/Beijing/32/92 and A/Singapore/6/86 (H1N1) viruses and influenza B/Panama/45/90 virus, according to the manufacturer's instructions. All other experimental details have been previously described (8). In brief, CD4⁺ T-cell lines (75, 88% CD4⁺) were selected for 3 weeks with a full-length HA sequence of influenza A/Beijing/32/92 virus. Detailed HA recognition was examined at days 14 and 21 with a panel of 16 amino acid peptides, overlapping by 11 residues, spanning the entire sequence of the A/Beijing/32/92 HA. Peptide proliferative assays were initially performed with pools of five peptides, and then responses were further localized with individual peptides. Peptides are numbered according to the N-terminal amino acid residue. Peptide pools are numbered by the N-terminal amino acid of the N-terminal peptide. Possible cross-reactivity among H3 influenza A virus HAs was investigated with HA derived from A/Aichi/68 (H3N2).

Prior to subunit vaccination, CD4⁺ T-cell lines derived from donors A to F expanded 0.6- to 2.6-fold in the presence of antigen (Table 1) and had A/Aichi/68 and A/Beijing/32/92 HA-specific proliferative responses of less than 12,000 cpm (Fig. 1). Two donors (C and E) mounted negligible responses to HA. In marked contrast, CD4⁺ T-cell lines derived from donors G and H expanded at least eightfold during culture and had higher levels of HA-specific proliferation (Fig. 2). Donor H had a history of influenza during November and December of 1993, whereas donor G had no recollection of an influenza-like illness during the past 5 years. We have previously reported a marked difference in the relative in vitro expansion of CD4⁺ T-cell lines derived from donors recently exposed to influenza (7.2-fold expansion during 21 days of culture) compared with those from adults with no recent history of influenza (1.1-fold expansion during 21 days of culture) (8). We therefore believe that donor G had a recent subclinical influenza virus infection.

Following vaccination, CD4⁺ T-cell lines derived from donors A to F showed increases in both in vitro cell expansion (Table 1) and A/Beijing/32/92 and A/Aichi/68 HA-specific proliferation (Fig. 1). Vaccination clearly boosted the proliferative responses of donors A to F to the HA peptide pools (Fig. 1). Serum from each donor recognized at least one of the pools representing the highly conserved HA2 subunit (residues 329 to 550). Furthermore, the majority of responses to the HA1 subunit localized to regions conserved among influenza A virus H3 strains (individual peptide data not shown), with only two major exceptions: donor E responded to residues 146 to 61, which included one conservative substitution between A/Aichi/68 and A/Beijing/32/92 (G-146 to S) and five nonconservative substitutions (T-155 to H, E-156 to K, G-158 to E, S-159 to Y, and T-160 to K), and donor F responded to residues 137 to 157, which included four conservative substitutions (S-143 to P,

FIG. 1. CD4⁺ T-cell lines were derived from donors A to F before (open columns) and 3 months following (striped columns) vaccination with Influvac. Cell lines were selected with the full-length HA sequence from influenza A/Beijing/32/92 (H3N2) virus. T-cell proliferation responses to A/Aichi/68 (H3N2) HA (1.0 μg/ml), A/Beijing/32/92 HA (0.1 μg/ml), and peptide pools (five peptides per pool; 10 μg of each peptide per ml) were tested at 14 or 21 days of culture with irradiated autologous prepulsed peripheral blood mononuclear cells as antigen-presenting cells. At 48 h, T-cell lines were pulsed with [^{3}H]thymidine and harvested 16 h later. The x axis represents the geometric mean of triplicate wells. Control (Cont), proliferative response of T-cell lines to complete medium and autologous antigen-presenting cells; Aichi, response of T-cell line to A/Aichi/68 HA (1.0 μg/ml); Beij, response to A/Beijing/32/92 HA (0.1 μg/ml). Peptide pools are labelled according to the number of the N-terminal residue of the N-terminal peptide in the pool (five peptides per pool; 10 μg of each peptide per ml).

S-145 to N, G-146 to S, and T-155 to H) and three nonconservative substitutions (N-137 to Y, G-144 to V, and E-156 to K) (7).

Donors G and H showed no marked increase in the cell expansion of their CD4⁺ T-cell lines following vaccination. The magnitudes of their anti-A/Aichi/68 and anti-A/Beijing/32/92 HA-specific proliferative responses did increase but were associated with a much smaller overall rise in the A/Beijing/32/92 HA-specific peptide response. In both donors, the HA2 subunit was recognized, and the great majority of their responses to HA1 were directed to conserved regions.

As the vaccine contained HA derived from an H1 influenza A virus, we were not able to directly examine the cross-reactivity of response with H1 and H2 influenza A viruses. However, because donors A, D, F, G, and H responded to residues 463 to 473 of HA and donors B and E responded to residues 442 to 462 and both regions are highly conserved among human influenza A virus subtypes, such cross-reactivity is likely.

Following natural infection we identified two conserved regions of HA, residues 303 to 323 and 407 to 428, which induced CD4⁺ T-cell responses from 17 of 18 unrelated donors, despite differences in their MHC class II haplotypes (8). Following subunit vaccination, these regions were recognized by 8 of 8 and 6 of 8 donors, respectively, though the magnitudes of individual donors' responses varied.

Clear MHC class II haplotype-associated recognition patterns of HA were evident. Donors A and G, who express identical DRB1* and DQ1* alleles, responded to the same peptide pools, despite differences in their prevaccination responses. Furthermore, donors D, E, and F, who share the DRB1*0101- and DQB1*05-containing MHC class II haplotype, responded to peptide pools 97, 183, 295, and 407. Our study of natural infection (8) included several donors with this haplotype, allowing a direct comparison. For the two groups, identical peptide pools were recognized, and the responses within these pools were localized to identical individual peptides or pairs of peptides (data not shown). Overall, the levels of proliferative response by the two groups were similar. We are currently investigating whether these results also reflect similar patterns of cytokine production.

In conclusion, influenza virus subunit vaccination enhances CD4⁺ T-cell recognition of conserved HA epitopes in a manner analogous to that observed following natural infection. At present influenza virus vaccines are offered to high-risk groups on an annual basis. It will therefore be important to monitor CD4⁺ T-cell responses after repeated annual vaccinations and to define the optimal interval between vaccinations for the maintenance of strong CD4⁺ T-cell responses to conserved HA epitopes.

We are most grateful to K. I. Welsh for help and advice regarding HLA typing, R. Brands and Solvay Duphar for the gift of highly purified A/Beijing/32/92 HA and Influvac, A. Hay and J. J. Skehel for the gift of A/Aichi/68 HA, and all our volunteer donors.

This study was supported by the Wellcome Trust.

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1.2 ANALYSIS OF THE ANTIGEN SPECIFICITY OF T CELL RESPONSES

1.2.2 MYCOBACTERIAL ANTIGENS

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INFECTION AND IMMUNITY, Oct. 1986, p. 177–183
0019-9567/86/100177-07$02.00/0

Vol. 54, No. 1

Immunological Activity of a 38-Kilodalton Protein Purified from *Mycobacterium tuberculosis*

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Received 27 January 1986/Accepted 18 June 1986

A 38-kilodalton (kDa) protein antigen from *Mycobacterium tuberculosis* was purified by monoclonal antibody TB71-based affinity chromatography. This molecule carries two nonoverlapping epitopes recognized by monoclonal antibodies TB71 and TB72, which are expressed substantially more strongly by *M. tuberculosis* than by *Mycobacterium bovis*. However, cross-reactive determinants between these two species were revealed on the 38-kDa protein by a rabbit anti-BCG serum. An immunoradiometric assay based on the TB71 and TB72 antibody pair specifically determined 38-kDa-antigen concentrations in mycobacterial extracts. Antibodies in sera from tuberculosis patients estimated by binding to 38-kDa-antigen-coated microtiter plates were positively correlated with TB72 competing titers. Unlike antibodies, T-cell proliferative responses to the 38-kDa protein were expressed equally by 60% of tuberculosis patients and healthy BCG-vaccinated subjects. Similarly, delayed-type hypersensitivity skin reactions were elicited in both *M. tuberculosis*- and *M. bovis*-sensitized guinea pigs. The results suggest the immunodominance of the species-specific B-cell and cross-reactive T-cell stimulatory epitopes.

Purified mycobacterial antigens are required as essential structural probes for dissecting the basic mechanisms of the immune response to mycobacteria. They also have potential applications as specific skin-test and serodiagnostic reagents. Although the immune reaction to crude mixtures of mycobacterial proteins such as tuberculin preparations has been extensively investigated over the past several decades, analysis of the activity of particular components has been frustrated by the technical difficulties encountered in identifying and purifying proteins from mycobacterial extracts (3). Antigens which were previously isolated from *Mycobacterium tuberculosis* include a 9.7-kilodalton (kDa) protein purified by ion-exchange chromatography (9), a 10-kDa protein purified by monoclonal antibody-based chromatography (11), and antigen 5 isolated by using a polyclonal antibody preparation (5). All these antigens were equally represented in *M. tuberculosis* and *M. bovis* and consequently no more specific as skin-test reagents than tuberculin.

The monoclonal antibody (MAb) technology opened the way toward the molecular definition of mycobacterial antigens. Two MAbs, TB71 and TB72, were reported to bind specifically to several strains of *M. tuberculosis* when compared with *M. bovis* and 20 other species of mycobacteria (4, 8). These two MAbs selectively discriminate in competition assays between the sera of patients with active pulmonary tuberculosis and the sera of control subjects (6, 7). In a recent comparative survey of 31 MAbs from nine separate laboratories, a total of seven distinct antigens were identified (H. D. Engers and V. Houba, Letter, Infect. Immun. 51:718–720). However, only two molecules, one of 38 kDa (identified by MAbs TB71 and TB72) and a 14-kDa protein, were reported to carry epitopes restricted to the *M. tuberculosis-M. africanum-M. bovis* complex. In view of these results and the previous data indicating that under appropriate experimental conditions antibodies to both identified epitopes of the 38-kDa antigen distinguished between *M. tuberculosis* and *M. bovis* (4, 8), we proceeded with the purification and characterization of this protein.

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MATERIALS AND METHODS

Bacteria. *M. tuberculosis* H37Rv was grown for 8 weeks as a surface pellicle on Sautons medium. Bacilli were removed by centrifugation and the culture supernatant was sterilized by passing it through a filter (0.45-μm pore size; Millipore Corp., Bedford, Mass.). Soluble extracts were prepared by disruption of 2.5 mrad ^{60}Co-irradiated organisms by using a Braun MSK cell disintegrator at 4,000 rpm for 2 min at 5 to 10°C. Bacterial debris was removed by centrifuging at 100,000 × *g* for 60 min, and the supernatant was passed through a filter (0.45-μm pore size) before it was applied to immunoaffinity columns.

Immunoaffinity chromatography. The globulin fraction precipitated by 18% Na_2SO_4 from the ascitic fluid of hybridoma TB71 was coupled to CNBr-activated Sepharose 4B (Pharmacia, Inc., Piscataway, N.J.) as recommended by the manufacturers by using a concentration of 7.5 mg of protein per ml of gel. The column (6 ml) was washed with phosphate-buffered saline (PBS) and elution buffers before use and stored at 0 to 4°C in PBS with 0.1% (wt/vol) sodium azide. Crude antigen (culture filtrate or soluble extract of *M. tuberculosis*) was passed through the column at room temperature with a flow rate of approximately 30 ml/hr, and the column was washed with PBS until the eluate had no detectable A_{280}. Bound material was then eluted with 4-column volumes each of a succession of three elution buffers, containing (i) 1.0 M NaCl, (ii) 0.1 M glycine–HCl (pH 2.5), and (iii) 10% (vol/vol) dioxan in 0.1 M glycine–HCl (pH 2.5). The column was then reequilibrated with PBS and stored. Fractions (5 ml) were collected during the elution procedure and assayed for 38-kDa protein by enzyme-linked

immunosorbent assay (ELISA) or by antigen capture assay. Fractions containing antigen were neutralized, dialyzed, and concentrated by freeze-drying. Protein concentrations were determined with bovine serum albumin (BSA) as the standard.

Antibody ELISA. The antigens were coated to Dynatech Immulon I microtiter plates (Dynatech Industries, Inc., McLean, Va.) by overnight incubation at 0 to 4°C in 0.1 M bicarbonate buffer (pH 8.3). The plates were washed three times with PBS (pH 7.2) containing 0.05% (vol/vol) Tween 20 (PBST), and 1% BSA in PBST was added for 30 min at 37°C to block nonspecific binding. Antibody samples (sera or MAbs) diluted in 1% BSA–PBST were added for a 1-h incubation at 37°C. The plates were washed three times with PBST, and peroxidase-conjugated secondary antibody was added for a further 1-h incubation at 37°C. Peroxidase-conjugated antibodies were affinity-purified goat anti-mouse immunoglobulin G (Bio-Rad Laboratories, Richmond, Calif.), affinity-purified goat anti-rabbit immunoglobulin G (Bio-Rad), and affinity-purified goat anti-human immunoglobulin G (gamma chain) (Sigma Chemical Co., St. Louis, Mo.) diluted in 1% BSA-PBST at 1:2,000, 1:3,000, and 1:1,000, respectively. After further washing with PBST, tetramethylbenzidine (dihydrochloride) (Aldrich Chemical Co., Inc., Milwaukee, Wis.) was added at 0.1 mg/ml in 0.1 M citrate buffer (pH 5) containing 0.03% H_2O_2. After 10 min at room temperature, the reactions were stopped by adding acid and the A_{450} was measured. The results were corrected for any color development in the absence of primary antibody or coating antigen. Hyperimmune rabbit anti-*M. bovis* BCG, anti-*M. paratuberculosis*, and anti-*M. duvalii* sera were obtained from Dako Ltd., High Wycombe, Buckinghamshire, United Kingdom.

Antigen detection by tandem immunoassay. MAb TB71 or TB72 was diluted to 10 μg/ml and coated to polyvinyl chloride microtiter plates by incubation for 2 h at 37°C. The plates were washed three times with PBST and blocked for 30 min with 1% BSA-PBST. Antigen samples diluted in 1% BSA-PBST were added to the wells followed by detector MAb (10^5 cpm per well) labeled with ^{125}I as described previously (6). After 1 h at 37°C, the plates were washed with PBST and bound radioactivity was determined in an LKB gamma counter (LKB Instruments, Inc., Rockville, Md.). The counts were corrected for any radioactivity bound in the absence of antigen or coating antibody.

Serum antibody competition test. Antibody titers inhibitory for the binding of ^{125}I-labeled TB72 and TB71 murine MAbs were determined essentially by the previously described technique (6). Briefly, polyvinyl chloride microtiter plates coated with the soluble extract of H37Rv were incubated with serial dilutions of human sera for 4 h followed by the addition of ^{125}I-labeled MAbs and further incubation for 20 h at 4°C. The radioactivities in the washed wells were corrected for background, and the 50% inhibitory serum dilutions were derived from graphic plots.

Immunoblot technique. Antigen samples were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis under reducing conditions by using a minigel electrophoresis system (Bio-Rad). After electrophoresis, the proteins were transferred to nitrocellulose by blotting for 1 h at 50 V (13). The nitrocellulose membranes were washed with 0.1% Triton X-100 in PBS, and nonspecific binding was blocked by incubation with 1% BSA in PBS-Triton. MAbs (2.5 μg/ml), rabbit anti-*M. bovis* BCG antibodies (1:10,000), or human sera (1:100) diluted with 1% BSA in PBS-Triton were added to the membranes, and they were incubated with shaking for 1 h at room temperature. After the membranes were washed with PBS-Triton, peroxidase-conjugated secondary antibodies were added as described above for the ELISA. Color development was accomplished by incubating washed membranes in diaminobenzidine dihydrochloride (0.1 mg/ml; Sigma) in 0.1 M citrate buffer (pH 5) with 0.03% H_2O_2. After 5 min, the membranes were thoroughly washed with deionized water and allowed to dry. For the localization of total protein, the nitrocellulose membranes were stained with amido black immediately after electroblotting. Alternatively, bound MAbs were detected with ^{125}I-labeled affinity-purified rabbit anti-mouse antibody. Autoradiographs were then prepared by exposing the washed membranes with X-ray films for 1 to 3 days at −70°C.

Human T-lymphocyte proliferation assay. Peripheral blood mononuclear leukocytes were isolated on a Ficoll-Hypaque density gradient and suspended (5×10^5/ml) in microtiter trays in RPMI-1640 supplemented with 2 mM L-glutamine, 100 IU of penicillin-streptomycin per ml, and 10% A^- human serum. The soluble extract of H37Rv, purified protein derivative (PPD) (Evan Medical Ltd., Greenford, Middlesex, England), or 38-kDa protein were added at the initiation of these cultures over a concentration range of 0.01 to 10 μg/ml. After 6 days of incubation, the cultures were pulsed with 1.0 μCi of tritiated methyl thymidine (^3H-TdR; Amersham International, Amersham, Buckinghamshire, United Kingdom) for 16 h and harvested onto glass fiber filters. Proliferation, as correlated with ^3H-TdR incorporation, was measured by liquid scintillation spectroscopy. The results are expressed as the mean count per minute ± standard error of the mean for triplicate cultures.

DTH assay. For the delayed-type hypersensitivity (DTH) assay, guinea pigs of the Hartley strain (female, 250 to 300 g) were sensitized with killed (2.5 mrad ^{60}Co-irradiated) BCG or soluble extracts of mycobacteria in incomplete Freund adjuvant by subcutaneous injection into three sites at the back of the neck. Skin reactions were elicited 26 days after sensitization by intracutaneous injection. Two diameters of induration at 90° were measured 24 h later. (For the dosages of used materials, see Table 4.)

RESULTS

Demonstration of two nonoverlapping epitopes (TB71 and TB72) on the 38-kDa protein from *M. tuberculosis*. A Western blot analysis of the reactivity of MAbs TB71 and TB72 with a soluble extract from *M. tuberculosis* indicated that both MAbs recognized a single band with a molecular weight of 38,000 (Fig. 1A). Each MAb was previously tested for its ability to compete with the binding of the other MAb in labeled antibody competition assays, and neither produced cross-competition, indicating that they were recognizing spatially separate epitopes (6). To confirm that the two epitopes were located on the same protein molecule, a tandem immunoassay was set up in which one antibody was bound to microtiter plates and used to capture the antigen, which was then detected by using the alternative antibody labeled with ^{125}I. The performance of the tandem assay with [^{125}I]TB72 (Fig. 2A) or [^{125}I]TB71 (Fig. 2B) as the detector antibody was compared by using soluble extracts from *M. tuberculosis* or *M. bovis* BCG as the source of the antigen. The positive results in this assay demonstrate that the two epitopes are indeed located on the same protein molecule. It is of interest that doses of *M. bovis* soluble extract at least 10 times higher than those of *M. tuberculosis* soluble extract

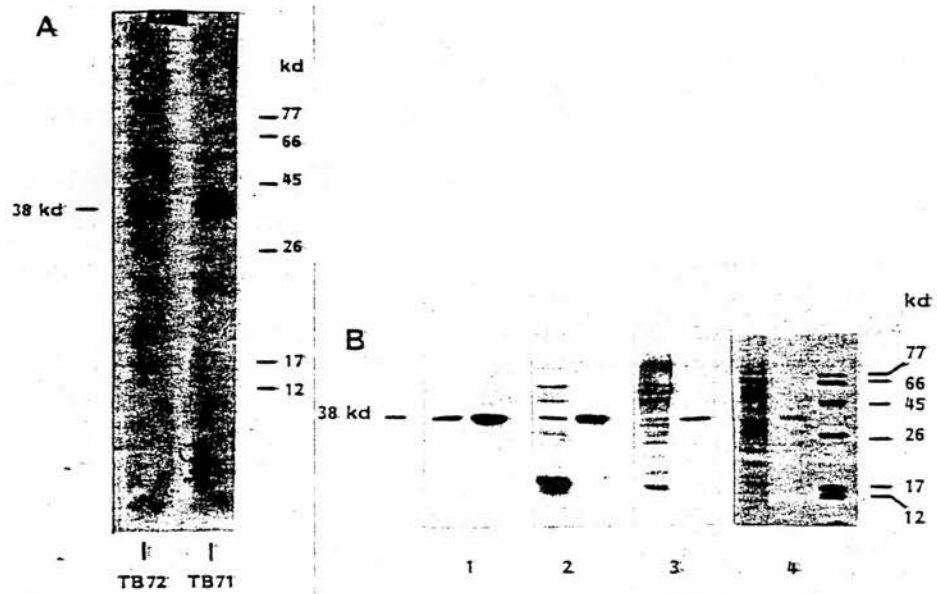

FIG. 1. Western blot analysis of the 38-kDa protein. (A) Soluble extract from *M. tuberculosis* H37Rv (10 μg per lane) was run in an SDS-polyacrylamide gel and transferred to nitrocellulose by electroblotting. Membranes were developed with MAbs TB71 and TB72 and then with a radiolabeled secondary antibody. (B) Soluble extract (10 μg) from *M. tuberculosis* H37Rv (left lane) and 0.3 μg of purified 38-kDa protein (right lane) were run in an SDS-polyacrylamide gel (12.5% acrylamide) and transferred to nitrocellulose by electroblotting. Membranes were developed with MAb TB71 (1), serum from a tuberculosis patient (2), rabbit anti-*M. bovis* BCG (3), or amido black staining (4).

were needed to obtain the same level of binding in the assay. This quantitative difference between *M. tuberculosis* and *M. bovis* is consistent with previous results concerning the specificities of these two MAbs (4, 8).

Isolation and characterization of the affinity-purified 38-kDa antigen. The outcome of the purification of a 30-ml sample of culture filtrate from *M. tuberculosis* H37Rv by immunoaffinity chromatography on TB71-Sepharose is summarized in Table 1. The 38-kDa-antigen content in the purification samples was estimated by using the tandem immunoassay with TB72 as the detector antibody. Antigen-containing samples were titrated in the assay, and the amount of the sample required to produce 50% of the maximum (plateau) binding level obtained by adding excess antigen in the assay was determined. Results are expressed in terms of protein concentrations based on use of a standard curve generated with the purified 38-kDa antigen in the tandem assay (Fig. 2C). The amount of the purified 38-kDa protein required to give 50% activity in the assay varied from 1 to 2 ng in different experiments using freshly isolated preparations. Repeated freezing and thawing of purified protein caused a reduction in antigenicity, with 3 to 5 ng required for 50%

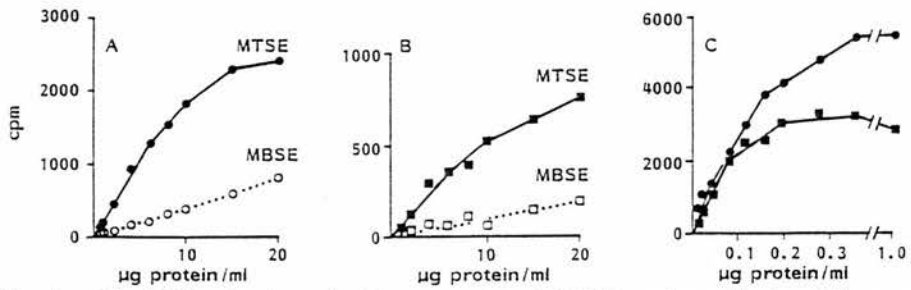

FIG. 2. Detection of the 38-kDa antigen by tandem immunoassay. (A and B) 38-kDa-antigen activity in soluble extracts from *M. tuberculosis* (MTSE, ●, ■) or *M. bovis* (MBSE, ○, □) with [^{125}I]TB72 (A) or [^{125}I]TB71 (B) as the detector MAb. (C) TB71 affinity-purified protein. ●, TB71 capture with [^{125}I]TB72 detector MAb; ■, TB72 capture with [^{125}I]TB71 detector MAb.

TABLE 1. Purification of 38-kDa protein by affinity chromatography on TB71-Sepharose

Fraction[a]	Vol (ml)	Total protein		38-kDa protein[b]		% Purity[c]
		mg/ml	Total mg	μg/ml	Total μg	
Starting material	30	1.38	41.4	14.0	420	1.01
Filtrate	100	0.37	37.0	0.6	60	0.16
Eluate	35	<0.05	0.3[d]	8.8	308	100.0

[a] The starting material was culture filtrate from *M. tuberculosis* H37Rv grown on Sautons medium. The filtrate fraction contained the material which passed through the column without binding along with the initial PBS wash. The 38-kDa antigen was eluted from the column by 10% dioxan in 0.1 M glycine–HCl buffer.

[b] The concentration of the 38-kDa protein was estimated by using the antigen capture assay with [^{125}I]TB72 as the detector antibody. The amount of pure protein required to give 50% activity in the assay was calculated as 5 ng from the standard curve shown in Fig. 2C.

[c] The 38-kDa protein as a percentage of the total protein present in each fraction.

[d] Determined after freeze-drying.

activity in the case of preparations stored for several months.

The content of the 38-kDa antigen per mg of protein was increased 100-fold during the purification procedure, with 74% of the original antigen recovered in the purified fraction eluted in the presence of 10% dioxan in glycine-HCl buffer (pH 2.5) (Table 1). Optimal results were obtained by immunoaffinity chromatography of culture filtrates of *M. tuberculosis*. The specific antigen contents of soluble extracts from *M. tuberculosis* were similar to those found in culture filtrates, but immunoaffinity chromatography was less satisfactory with such preparations, with less than 50% of the antigen binding to the column even after repeated passage.

The purity of the 38-kDa antigen preparation was investigated by using SDS-polyacrylamide gel electrophoresis along with a variety of detection methods. Direct silver staining of gels (2) or amido black staining of nitrocellulose blots showed a single band of protein with a molecular weight of 38,000 (Fig. 1B, panel 4). Similarly, autoradiography of gels run with antigen labeled with ^{125}I showed a single band at the same molecular weight. To screen for low amounts of antigenic contaminants, Western blots of the purified antigen were screened with polyvalent antisera having high activities against mycobacterial antigens. Examples of such blots with sera from a tuberculosis patient and a rabbit hyperimmunized with *M. bovis* BCG are shown in Fig. 1B (panels 2 and 3). Although extensive reaction with multiple molecular weight bands in crude *M. tuberculosis* extracts was observed (Fig. 1B, panels 2 and 3, left lanes), only a single band of reactivity was seen with the purified antigen (right lanes). Analysis of purified antigen preparations with MAbs recognizing proteins with molecular weights at 65,000, 23,000, 19,000, and 14,000 (4; Engers and Houba, Letter, 1986) failed to show detectable levels of contaminating protein in an ELISA or a dot blot (14) assay.

TABLE 2. Analysis of 38-kDa protein with polyclonal rabbit antisera

Rabbit serum	Maximum A_{450} with[a]:		Dilution of antiserum (10^3) giving an A_{450} of 0.3 with:	
	MTSE[b]	38-kDa protein	MTSE[b]	38-kDa protein
Anti-*M. bovis* BCG	0.70	0.50	200	10.0
Anti-*M. paratuberculosis*	0.70	<0.01	200	NA[c]
Anti-*M. duvalii*	0.75	<0.01	200	NA
Nonimmune	0.30	<0.01	0.2	NA

[a] Antisera were tested in an ELISA, as described in the text, at dilutions ranging from 1:200 to 1:625,000.

[b] Soluble extract from *M. tuberculosis* H37Rv.

[c] NA, Not applicable.

TABLE 3. Proliferative responses to the 38-kDa protein in tuberculosis patients and healthy controls[a]

Subjects	No. tested	SI		
		>4	2 to 4	<2
Tuberculosis patients	10	6	1	3
Healthy controls	18	11	2	5

[a] Peripheral blood mononuclear cells (2×10^5/ml) were cultured for 7 days with a range (0.01 to 10.0 μg/ml) of 38-kDa protein concentrations. The maximal proliferative response was obtained for each donor from the dose-response curve. The SI was calculated by using this value and the counts per minute obtained in unstimulated cultures. A similar distribution was also obtained if responsiveness was expressed as the difference in maximum stimulated counts per minute minus the counts per minute in unstimulated controls (Δcpm) and ranked as high (Δcpm, <5,000), medium (Δcpm, 2,000 to 5,000), and low (Δcpm, <2,000) responsiveness.

Recognition of the 38-kDa antigen by anti-mycobacterial antisera was further investigated by testing rabbit antisera to *M. bovis* BCG, *M. paratuberculosis*, and *M. duvalii* in ELISAs with *M. tuberculosis* soluble extract or purified 38-kDa protein as the coating antigen. All of the antisera bound to the crude extract with comparable reactivities in terms of maximum absorbance and serum titer, whereas only the anti-*M. bovis* BCG serum reacted with the 38-kDa protein (Table 2).

Application of purified 38-kDa antigen for serological testing of human sera. Sera from 14 patients with active pulmonary tuberculosis were examined for binding to 38-kDa-antigen-coated plates (Fig. 3). The sera were ranked according to the binding value (at an optical density at 450 nm

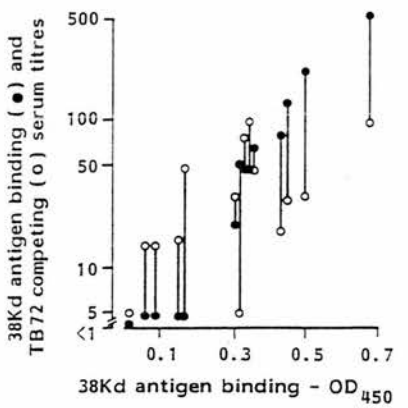

FIG. 3. Detection of 38-kDa-antigen-binding antibodies by ELISA. Microtiter plates coated with 0.05 μg of 38-kDa protein were reacted with human sera and peroxidase–anti-human immunoglobulin G conjugate. *y* axis: binding titers represent serum dilutions giving 0.2 OD_{450}; competing titers represent 50% inhibitory serum dilutions. TB71 50% inhibitory serum dilution, >1/10. *x* axis: 38-kDa-antigen-binding OD_{450} at a fixed 1/5 serum dilution.

[OD_{450}]) at a 1/5 serum dilution on the x axis, and the quantitatively determined 38-kDa binding titers and [^{125}I]TB72 competing titers (i.e., 50% inhibitory serum dilutions) on the y axis were compared. The 38-kDa protein-binding antibody titers of most tested sera were positively correlated with the TB72-competing titers. Only one 38-kDa-binding positive (titer, 50) serum had TB72 competing activity 10 times lower. Conversely, three sera with less than 0.2 OD_{450} values showed significant (titers, 14 to 15) TB72 competition. These results suggest that the antibody response of tuberculosis patients is directed predominantly against the TB72 epitope of the 38-kDa antigen. Only three sera (21%) revealed TB71-competing activity.

T-cell recognition of 38-kDa protein. The proliferative response of peripheral blood T lymphocytes to the 38-kDa protein was determined. A positive response (stimulation index [SI], > 4) was obtained with lymphocytes from 6 of 10 patients with active pulmonary tuberculosis and also with those from 11 of 18 individuals vaccinated with BCG but having no clinical history of tuberculosis (Table 3). The remaining subjects produced either no stimulation (SI, <2) or a marginal increase of the lymphocyte response (SI, 2 to 4). Thus, the frequency of significant lymphocyte responses to the 38-kDa antigen was similar in tuberculosis patients and BCG-vaccinated controls. Examples of positive and negative responses to the 38-kDa protein by tuberculosis patients are shown in Fig. 4A and B. The 38-kDa protein was required at a concentration of 3 μg/ml to induce maximal stimulation, equivalent to the plateau elicited by PPD and H37Rv. Increasing the concentration of the 38-kDa protein to 10 μg/ml resulted in a mild reduction of proliferation, in contrast to the increased activation induced by PPD and H37Rv at that concentration. Furthermore, at concentrations of <0.1 μg/ml, both PPD and H37Rv stimulated T-cell proliferation, whereas the 38-kDa protein failed to do so (Fig. 4A). While the 38-kDa protein over the concentration range tested (0.01 to 10.0 μg/ml) failed to stimulate the T lymphocytes of the nonresponder population (Fig. 4B), PPD and H37Rv induced proliferation, with an overall pattern similar to that observed in the responder population (Fig. 4A).

Skin DTH response of guinea pig. The potency of the 38-kDa protein to evoke a skin DTH reaction was determined in guinea pigs presensitized with BCG or with soluble extracts from either *M. tuberculosis* or *M. bovis*. The results showed weak reactions to the 38-kDa antigen, 4.0 to 6.0 mm mean diameter, as compared with 8.8- to 10.5-mm reactions to PPD and 13.0- to 17.7-mm reactions to *M. tuberculosis* soluble extract. The positive reactions in the *M. bovis*-sensitized groups suggested that cross-reactive epitopes of the 38-kDa protein were recognized by DTH-effective T cells.

DISCUSSION

Purification of protein antigens either from mycobacterial extracts as described here or possibly from *Escherichia coli* containing recombinant DNA (15) is a prerequisite for the detailed analysis of the role of each of these components in the immune response to mycobacteria. Immunoaffinity chromatography using MAb TB71 provided a simple one-step purification procedure for the 38-kDa protein of *M. tuberculosis*. The successful outcome could have been predetermined by the lack of complexing of the antigen with other molecules in the starting preparation, as well as by the suitable affinity of the used TB71 MAb. The use of high-salt-concentration and low-pH buffers before elution of the antigen with a hydrophobic solvent at low pH probably contributed to the purity of the final preparation.

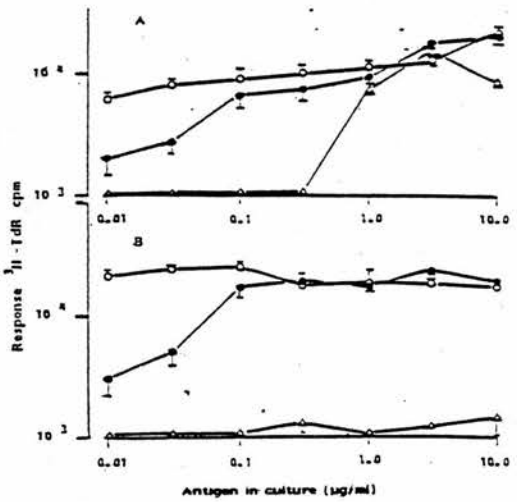

FIG. 4. Stimulation of human lymphocytes with the 38-kDa protein. Peripheral blood mononuclear cells of a responder (A) and nonresponder (B) at 2×10^5/ml were stimulated with either *M. tuberculosis* soluble extract (●) PPD (○), or the 38-kDa protein (△) at a range of concentrations. Proliferation as correlated with ^{3}H-TdR incorporation was determined after 7 days of culture. The results are expressed as the mean counts per minute ± the standard error, with the counts per minute of the control unstimulated cultures subtracted. The background response of the control cultures was less than 2,500 cpm. The peripheral blood mononuclear cells were tested for in vitro proliferative responses 7 days after the addition of antigen by the incorporation of ^{3}H-TdR. The results are expressed as the mean counts per minute ± the standard error minus the counts per minute (2,144 ± 198) incorporated in control unstimulated peripheral blood mononuclear cells.

It seemed mandatory for the subsequent analysis of specificity and immunological activity of the 38-kDa protein to ascertain the purity of the obtained preparation. Compelling evidence demonstrating that the obtained preparation was substantially free of other antigenic components was provided by the immunoblot technique by using a hyperimmune polyspecific antiserum to *M. bovis* BCG. This antiserum, directed toward multiple antigens in *M. tuberculosis* soluble extract, detected only a single 38-kDa band in the affinity-purified material. Furthermore, no significant binding was revealed by immunoassay with MAbs ML30 and ML34 directed to antigens which were contaminating previously reported affinity-purified fractions (8).

The binding of both TB71 and TB72 MAbs to the TB71 affinity-column-eluted fraction showed unequivocally that they represent two distinct epitopes on the 38-kDa molecule. Consequently, it was possible to use this pair of MAbs as capture and detector reagents, respectively, in a tandem immunoassay. This assay is eminently suitable for the specific quantitative estimation of 38-kDa-antigen concentrations in crude bacterial extracts. There is a particular advantage in this technique as compared with a coating of antigen onto solid phase, in which binding values can be masked by

other abundant antigens (Engers and Houba, Letter, 1986). Comparative analysis of soluble extracts from *M. tuberculosis* and *M. bovis* BCG by the tandem assay clearly demonstrated at least 10-fold-higher expression of both the epitopes of the native 38-kDa protein in *M. tuberculosis*. This is consistent with the original solid-phase immunoassay results (4) and with the previous demonstration of weak *M. bovis* cross-reactivity by methods which involved a certain degree of protein denaturation (8; Engers and Houba, Letter, 1986). However, the presence of a 38-kDa homologous protein in *M. bovis* was suggested by the presence of cross-reactive antibodies in a hyperimmune rabbit anti-*M. bovis* BCG serum. These antibodies may be directed to cross-reactive determinants which are either distinct or sterically related to the TB71 and TB72 epitopes. However, hyperimmune antisera to *M. paratuberculosis* and *M. duvalii* of comparable titer to the anti-*M. bovis* serum did not bind to the 38-kDa protein.

Previous analysis of the antibody repertoire in active pulmonary tuberculosis by the serum competition test showed activity toward the TB72 epitope in the majority of patients and activity toward the TB71 epitope in the minority, but neither was present in healthy BCG-vaccinated controls (6). In the present study, it was of interest to examine human sera antibodies for binding to purified 38-kDa-antigen-coated polyvinyl chloride plates which seemed to offer possible technical advantages over the competition test. The results of a pilot study on sera from 14 tuberculosis patients showed a positive correlation between TB72 competition titers and total 38-kDa-protein binding. This is consistent with the conclusion that in tuberculosis patients the TB72 epitope is the main immunodominant epitope of the 38-kDa antigen. Thus, it is not surprising that marginally positive sera could be detected with greater sensitivity by the TB72 competition test. However, more extensive evaluation of the 38-kDa-antigen-based assay for the serology of tuberculosis is required.

In view of the presence of the two serologically active *M. tuberculosis*-specific epitopes on the 38-kDa protein, it was of considerable interest to examine the T-cell stimulatory activity of the affinity-purified antigen in humans and in guinea pigs. Unlike B-cell responses, the T-cell-proliferative in vitro assay was stimulated by the 38-kDa antigen in about equal proportions (60%) of tuberculosis patients and healthy BCG-vaccinated controls. Furthermore, DTH skin reactions elicited by the 38-kDa antigen were not significantly different when guinea pigs sensitized with *M. tuberculosis* and *M. bovis* were compared. The relatively high 38-kDa-antigen concentration (when compared with that of PPD) required for the optimal stimulation of human lymphocytes could have applied also for guinea pigs and therefore may explain their low skin-test response. Nevertheless, this result does not need to imply a weak immunogenicity of the 38-kDa antigen, considering that the superior potency of PPD is probably due to its complexity of antigens which stimulate T cells of multiple specificities. Taken together, these results suggest that the immunodominant T-cell stimulatory determinants of the 38-kDa protein are shared between *M. tuberculosis* and *M. bovis*. The observation that the immunodominant determinant for the antibody response appears to be species-specific whereas the immunodominant T-cell determinant is cross-reactive with *M. bovis* suggests that the 38-kDa protein may be useful as a diagnostic reagent for serological testing but is unlikely to be a specific skin-test reagent. Information on any protective effect of the T-cell response to the 38-kDa protein, and consequently any potential application towards vaccine development, is an important subject for further study.

Although it is generally accepted that B and T lymphocytes recognize distinct structural determinants on antigens (10, 12), so far little is known about the pairing of the respective constitutive epitopes on proteins (1). These relationships are not at all understood when the antigens are parts of an even more complex bacterial structure. However, the obtained data indicate that the B-cell responses toward the *M. tuberculosis*-specific epitopes of the 38-kDa antigen probably use cross-reactive helper T cells. Finally, it is conceivable that the search for truly specific reagents for skin testing will require the use of peptides containing single epitopes of desired specificity rather than whole protein molecules.

ACKNOWLEDGMENTS

Peripheral blood from tuberculosis patients was kindly provided by R. Rudd, London Chest Hospital, and G. Bothamley, West Middlesex Hospital. The samples from healthy controls were provided by M. Contreras from the National Blood Transfusion Centre, Edgeware, and by laboratory personnel.

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The EMBO Journal vol.6 no.5 pp.1245–1249, 1987

Mapping of T cell epitopes using recombinant antigens and synthetic peptides

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Communicated by J.H.Humphrey

Two complementary approaches were used to determine the epitope specificity of clonal and polyclonal human T lymphocytes reactive with the 65-kd antigen of *Mycobacterium leprae*. A recombinant DNA sublibrary constructed from portions of the 65-kd gene was used to map T cell determinants within amino acid sequences 101–146 and 409–526. Independently, potential T cell epitopes within the protein were predicted based on an empirical analysis of specific patterns in the amino acid sequence. Of six peptides that were predicted and subsequently synthesised, two (112–132 and 437–459) were shown to contain human T cell epitopes. This corroborated and refined the results obtained using the recombinant DNA sublibrary. Both of these regions are identical in *M. leprae* and *M. tuberculosis* and are distinct from the known B cell epitopes of the 65-kd protein. This combination of recombinant DNA technology and peptide chemistry may prove valuable in analysis of the cellular immune response to infectious agents.

Key words: epitopes/mycobacteria/peptides/recombinant DNA/T cells

Introduction

Identification of antigenic determinants within protein molecules is of theoretical importance in understanding the fundamental interactions involved in immune responses and of potential practical value in the design of 'subunit' vaccines and specific diagnostic reagents. Information has accumulated recently on the architecture of both antibody binding sites and T cell epitopes for a number of structurally well-defined proteins such as lysozyme and myoglobulin (Benjamin *et al.*, 1984) and considerable interest has been shown in the use of synthetic peptides for modulation of antibody responses to infectious agents (Lerner, 1984). Several predictive methods have been described for the identification of potential B cell epitopes (Hopp and Woods, 1981; Tainer *et al.*, 1984; Westhof *et al.*, 1984; Barlow *et al.*, 1986). Because an effective immune response to many pathogens, such as intracellular parasites, is dependent on T cell activation (Hahn and Kaufman, 1981), it is also necessary to develop strategies to identify T cell determinants. The observation that T cell determinants generally consist of short linear peptide sequences has stimulated the development of predictive theories for T cell epitopes that are based on analysis of amino acid sequence (DeLisi and Berzofsky, 1985; Rothbard, 1986).

Pathogenic mycobacteria are the cause of widespread chronic diseases, particularly in developing countries, with an estimated 30 million individuals suffering from tuberculosis and a further 10–15 million individuals with leprosy (Bloom and Godal, 1983). The outcome of mycobacterial infection is thought to be determined by the interaction of mycobacterial antigens with T cells (Mackaness, 1964) and recent studies have identified some of the mycobacterial proteins which are involved in recognition by human T cells (Emmrich *et al.*, 1986; Mustafa *et al.*, 1986; Ottenhoff *et al.*, 1986; Young *et al.*, 1986; Lamb and Young, 1987; Oftung *et al.*, 1987).

The gene for a major mycobacterial antigen, the 65-kd protein, has been cloned from *Mycobacterium tuberculosis* (Young *et al.*, 1985a; Husson and Young, 1987) and from *M. leprae* (Young *et al.*, 1985b). Sequence data shows that this antigen is highly conserved in the two mycobacteria (Mehra *et al.*, 1986; Shinnick, 1987). The 65-kd protein is recognised by a high proportion of the many different murine monoclonal antibodies raised against mycobacterial extracts (Engers *et al.*, 1985,1986), suggesting that the antigen is important in the B cell response to mycobacteria. A recombinant DNA expression method has been used to map the epitopes recognised by six of these monoclonal antibodies (Mehra *et al.*, 1986).

The 65-kd antigen is also involved in the T cell response to infection, and T cell clones from patients with tuberculosis or leprosy have been shown to proliferate in response to the 65-kd protein (Emmrich *et al.*, 1986; Lamb *et al.*, 1986; Oftung *et al.*, 1987). However, the precise location of the T cell determinants within the molecule has not yet been established.

In this paper we have exploited two new techniques to map human T cell epitopes within the 65-kd protein, one based on recombinant DNA expression (Mehra *et al.*, 1986) and the other based on the synthesis of peptides whose sequences are predicted to constitute T cell determinants (Rothbard, 1986).

Results

Expression of antigens by recombinant DNA clones

Construction of a set of recombinant DNA clones containing overlapping fragments of the structural gene of a protein can be used to determine the location of antigenic sites within the molecule. Screening of such a sublibrary with monoclonal antibodies has been used to identify six B cell epitopes in the 65-kd protein (Mehra *et al.*, 1986) and we have used an analogous approach to locate T cell determinants. Figure 1 illustrates the regions of the gene covered by each of the recombinant DNA clones.

Expression of antigens in lysogenic strains derived from the phage clones listed in Figure 1 was analysed by Western blotting as shown in Figure 2. The phage clone which was used for construction of the sublibrary (Y3178) contains a DNA insert coding for the entire 65-kd protein of *M. leprae* but with an orientation reversed with respect to the *lacZ* gene (Mehra *et al.*, 1986). The antigen expressed by this clone has a mol. wt of ~65 kd and is clearly not fused to β-galactosidase (lane 8). An analogous

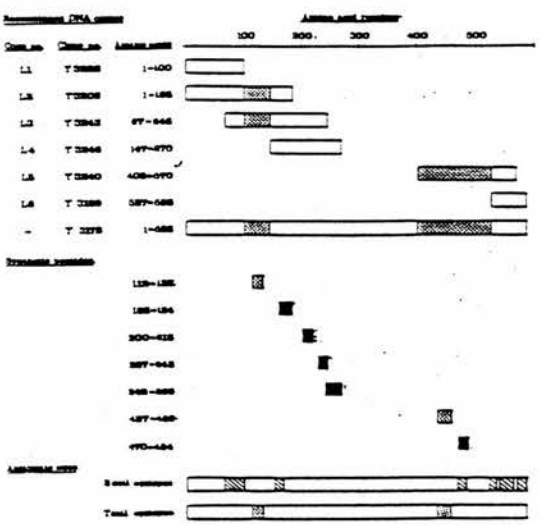

Fig. 1. Map showing areas of the 65-kd protein covered by DNA sublibrary clones and synthetic peptides. The amino acid residues of the 65-kd protein (1–588, based on proposed *M. leprae* coding sequence) are shown on the scale along the top. The upper portion of the diagram shows the areas of the gene covered by the DNA sublibrary clones, while the lower portion shows the synthetic peptides. The stippled areas correspond to the regions recognised by T cells.

Fig. 2. Expression of mycobacterial antigen by recombinant DNA clones. Lysogens prepared in *E. coli* Y1089 using the λgt11 phages shown in Figure 1 were grown and induced as described in the text. Western blots were prepared and developed with monoclonal antibodies C1.1 (lanes 1–4), IIH9 (lane 5) and IIC8 (lanes 6–8). Lane 1 contained lysogen from clone Y3150; lane 2, Y3223; lane 3, Y3203; lane 4, Y3243; lane 5, Y3246; lane 6, Y3240; lane 7, Y3189; lane 8, Y3178. On the right-hand side of the figure is shown the migration position of standard protein markers with mol. wts (in kd) as indicated.

situation exists for clone Y3150 which expresses the 65-kd protein of *M. tuberculosis* (lane 1). In contrast, all of the sublibrary clones used in this study have insert DNA oriented with the *lacZ* gene (Meitra *et al.*, 1986) and Western blot analysis demonstrates that in each case the mycobacterial antigenic determinant is expressed as a high mol. wt fusion protein linked to β-galactosidase (Figure 2, lanes 2–7).

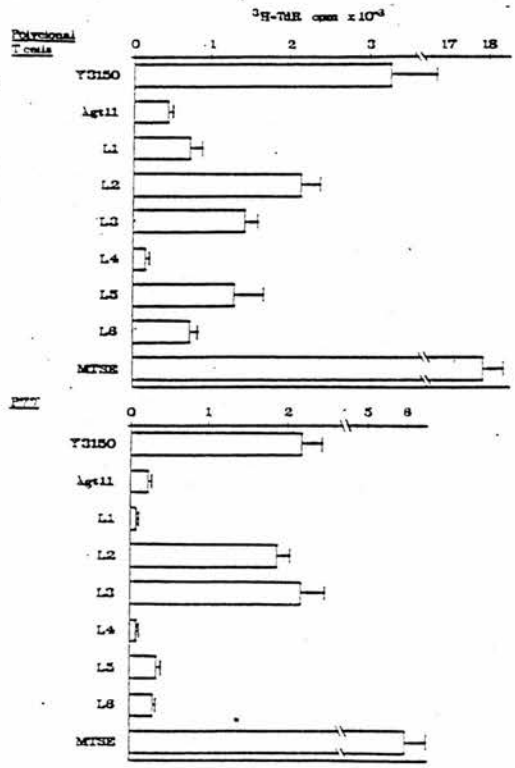

Fig. 3. T cell recognition of antigens expressed by recombinant DNA clones. Unfractionated ascitic lymphocytes were stimulated with extracts from Y3150, λgt11 (control lysogen with no insert DNA), L1, L2, L3, L4, L5, L6 (all at 30 μg protein/ml) and MTSE (1 μg protein/ml). Proliferation as correlated with [^{3}H]TdR incorporation was determined at day 6. The results are expressed as mean c.p.m. ± SEM of triplicate cultures. The response of T cells to medium alone was 575 ± 10 c.p.m. ± SEM. T cells of clone P77 were stimulated with the same antigens as above in the presence of autologous irradiated PBMC as a source of APC. Proliferation was determined at 72 h. The control response of P77 to APC in the absence of antigen was 325 ± 4 c.p.m. ± SEM.

After ultrasonication, the fusion protein from each clone was found to be present in approximately equal amounts in the soluble and insoluble fractions. Addition of ammonium sulphate to the soluble fraction (final concentration, 50% saturation at 0–4°C) resulted in complete precipitation of fusion proteins as judged by Western blot analysis. Previous experiments (data not shown) have indicated that *E. coli* extracts have a non-specific inhibitory effect on T cell proliferation assays which can be substantially decreased by this simple ammonium sulphate fractionation step.

T cell recognition of fusion proteins from 65-kd sublibrary

Products expressed by the intact 65-kd gene (Y3150) and the sublibrary clones L2 (residues 1-185), L3 (67–246) and L5

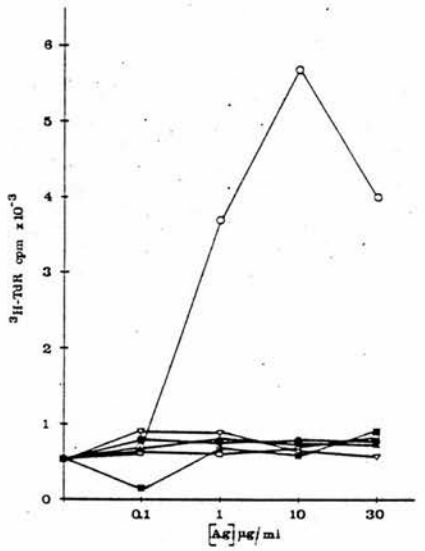

Fig. 4. Response of T cell clone P77 to synthetic peptides of 65-kd antigen. T cells of clone P77 were stimulated with peptides 112–132 (○), 163–184 (▽), 200–218 (□), 227–243 (▲), 242–266 (●), 437–459 (■) in the presence of autologous irradiated EBV-B cells. Proliferation was determined at 72 h.

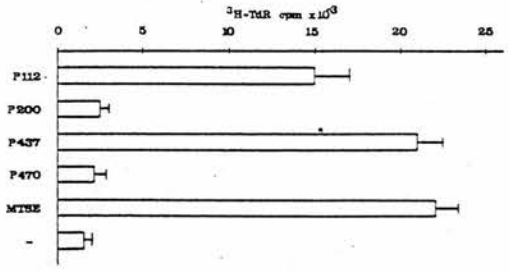

Fig. 5. Polyclonal T cell response to synthetic peptides of 65-kd antigen. Unfractionated ascitic lymphocytes were stimulated with peptides 112–132, 200–218, 437–459, 470–485, MTSE or alone in medium. Proliferation was determined at day 6. The results show the maximum proliferation of a dose–response curve for each antigen.

(408–570) induced a proliferative response in the polyclonal T cells of the ascitic effusion as compared to the control of a lysogen prepared using λgt11 without insert DNA (Figure 3). The response to L1, L4 and L6 was <2-fold higher than that of the background control of the λgt11 lysogen. This suggested that at the polyclonal level at least two regions of the 65-kd protein (residues 101–146 and 409–526) contained T cell epitopes (Figure 1). The response of the ascitic T cells to MTSE served as a positive control (Figure 3).

The pattern of recognition of the 65-kd sublibrary fusion proteins by T cells of clone P77, isolated from the ascitic effusion and previously reported to be specific for the 65-kd antigen (Lamb *et al.*, 1986), was also determined. Clone P77 proliferated in response to L2 and L3 but not to the adjacent sequences L1 and L4. In contrast to the response observed with ascitic T cell population, clone P77 was not stimulated by extracts from the recombinant clones covering the carboxyl terminus of the protein (L5 and L6) (Figure 3). The cloned T cells responded to the intact 65-kd protein (Y3150) and to MTSE but not to the control extract from a lysogen prepared from λgt11 without insert DNA. This suggests that the epitope recognised by P77 is located within residues 101–146.

112 132
YEKIGAELVKEVAKKTDDVAG

437 459
RKHRIEDAVRNAKAAVEEGIVAG

Fig. 6. Amino acid sequence of residues 112–132 and 437–459 of the 65-kd protein. Amino acids are represented by the single letter code. The underlined residues represent the characteristic patterns associated with the presence of predicted T cell epitopes (Rothbard, 1986).

Mapping of T cell epitopes with synthetic peptides

A high proportion of the peptides which have been reported to function as T cell determinants contain a distinctive pattern within their amino acid sequence (Rothbard, 1986). This pattern consists of a charged, or a glycine residue, followed by two or three consecutive hydrophobic residues, and terminating with a polar amino acid. The 65-kd antigen was analysed for the presence of such sequences and six peptides containing patterns similar to those in previously defined epitopes were identified and synthesised. Figure 4 lists the six peptides along with a control peptide (470–484) which contains a known B cell epitope (Mehra *et al.*, 1986) but lacks the characteristic T cell pattern.

Each peptide was tested with both the T cell clone and with the polyclonal population using the proliferation assay. T cells of clone P77 proliferated in a dose-dependent manner when stimulated with peptide 112–132 in the presence of antigen presenting cells, but not with the control peptides 163–184, 200–218, 227–243, 242–266 and 437–459 all from the 65-kd protein (Figure 4). The proliferation was equal to that elicited by preparations containing the intact protein or any of the fragments produced by the sublibrary clones, thus allowing us to conclude that this peptide did indeed contain the epitope for clone P77.

When the response of the uncloned T cells to the peptides was analysed, the same peptide (112–132) was found to be stimulatory and there was also a substantial response to the peptide 437–459 (Figure 5). By means of six peptides we were able to identify two distinct regions of the 65-kd protein that were stimulatory for the ascitic T cells of the donor selected in this study. These two peptides are located within the same two regions of the protein which were identified as immunostimulatory by the recombinant sublibrary approach. The amino acid sequences of the two stimulatory peptides are shown in Figure 6, with the residues corresponding to the predictive patterns underlined.

Discussion

The approach used here for mapping of epitopes recognised by T cells using a λgt11 sublibrary prepared from the gene coding

for the 65-kd antigen is analogous to that previously used to identify monoclonal antibody binding sites on the same protein (Mehra *et al.*, 1986). Although recognition of antigens in lysates from *Escherichia coli* by T cell clones has been reported (Lamb *et al.*, 1986; Mustafa *et al.*, 1986) we have been able to extend this technique to identify regions within a molecule which stimulate proliferation of both clonal and uncloned T cells. It is important to note that both 'free' antigens and antigens expressed as β galactosidase fusion proteins were recognised in these assays, although it is conceivable that a fusion point very close to the T cell epitope could cause a 'false negative' result if it leads to an alteration in antigen processing mechanisms. The polyclonal T cell population used in this study was obtained from an ascitic effusion and is highly enriched in T cells reactive with mycobacteria. Proliferation of polyclonal T cell preparations in response to *E. coli* lysates is quite variable; only preparations with strong responses to specific antigen and low background responses to *E. coli* itself will be suitable for this type of analysis.

The second strategy we have used to identify T cell epitopes is to test the ability of peptide antigens directly to stimulate T cells. Rothbard (1986) has identified a common pattern in the amino acid sequence of peptides containing known T cell epitopes and it is possible that the presence of such a pattern can be used to identify novel T cell epitopes. As a prototype, we have used the 65-kd antigen to test the possibility of using this approach to predict previously undefined T cell epitopes in a relatively large open reading frame. Because the known T cell epitopes are still quite small in number, the power of such an empirical prediction must, however, remain limited at the present time. Nonetheless we were able to identify two stimulatory epitopes in a polyclonal population by synthesising six peptides containing predicted T cell epitopes without knowledge of the results obtained using the recombinant DNA sublibrary. The two stimulatory peptide sequences fell within the regions of the protein identified by sublibrary mapping.

Both sequences 112–132 and 437–459 of the 65-kd protein are identical in *M. leprae* and *M. tuberculosis* (Mehra *et al.*, 1986; Shinnick, 1987) and this is consistent with the cross-reactivity observed at the clonal level for T cells specific for this protein (Emmrich *et al.*, 1986; Lamb *et al.*, 1986). Using mouse monoclonal antibodies, both species-specific and cross-reactive B cell epitopes have been identified on the 65-kd antigen (Mehra *et al.*, 1986) and it is interesting to note that none of these B cell epitopes overlap with the two T cell epitopes described here. Previous analysis of other protein antigens also suggests that T and B cell epitopes within a molecule need not overlap (Lamb and Green, 1983; Benjamin *et al.*, 1984). Whether T cells binding to the cross-reactive epitopes are able to function as helper cells to stimulate production of antibodies to adjacent specific or cross-reactive B cell epitopes remains to be investigated.

Whether the two regions of the 65-kd protein within which we have identified T cell epitopes are immunodominant at the population level or reflect the repertoire of restriction elements that this individual is able to use requires further investigation. The latter has been found to be true for the human cytotoxic T cell response to influenza virus where residues 335–349 of nucleoprotein are recognised only in the context of HLA-B37 (McMichael *et al.*, 1986). Each of the peptides contains two sets of predictive patterns as shown by the underlined residues in Figure 6 (118–121/122–125 and 443–446/449–453). It may therefore be necessary to test the effect of amino acid substitution at particular residues in order to analyse the association of these peptides with HLA-encoded determinants.

The experimental observations reported here show that a recombinant DNA sublibrary can be used for initial localisation of T cell epitopes within a protein. Determination of the nucleotide sequence of sublibrary clones allows derivation of an amino acid sequence which can be analysed for identification of potential T cell epitopes. The complementary strategy of using predicted peptide antigens verified and refined the results obtained using the recombinant DNA sublibrary. The combination of the two techniques leads to a rapid identification of the immunologically important sites within protein antigens. This approach to analysis of T cell recognition of the 65-kd protein from mycobacteria may be generally applicable to the investigation of other infectious agents and may allow production of simple defined peptide antigens which can be used as probes for detailed analysis of cellular immune responses.

Materials and methods

Antigens

Mycobacterium tuberculosis H37RV was grown for 8 weeks as a surface pellicle on Sauton's medium. A soluble extract (MTSE) was prepared by disruption of cobalt-irradiated organisms using a Braun MSK cell disintegrator at 4000 r.p.m. for 2 min at 5–10°C. Bacterial debris was removed by centrifugation at 30 000 *g* for 1 h, and supernatant material filtered through a 0.45-micron filter. Protein concentrations in the soluble extracts were determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Preparation of antigen from recombinant DNA clones

Preparation and characterisation of a λgt11 sublibrary from the DNA insert of clone Y3178 (*M. leprae* 65-kd antigen) has been described previously by Mehra (1986). The sublibrary clones used in the present study are shown in Figure 1 along with their relationship to the amino acid sequence of the 65-kd protein as deduced from the insert endpoint analysis (Mehra *et al.*, 1986). Other recombinant clones used in this study were Y3178 (*M. leprae* 65-kd antigen; Young *et al.*, 1985b) and Y3150 (*M. tuberculosis* 65-kd antigen; Young *et al.*, 1985a).

Lysogens were prepared from phage clones in *E. coli* Y1089 as described by Huynh *et al.* (1985). After growth in L-broth at 37°C to an absorbance of 0.5 at 600 nm, lysogens were induced by incubation for 20 min at 45°C, followed by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to 10 mM and further incubation at 38°C for 1 h. Cells were harvested by centrifugation, resuspended in one-tenth volume of phosphate-buffered saline (PBS), and lysed by sonic disruption using a Rinco B1070 ultrasonic probe at a maximum output for 20 s. Bacterial debris was removed by centrifugation at 10 000 *g* for 20 min, and an equal volume of saturated ammonium sulphate solution was added with vigorous mixing to the supernatant fractions. After 1 h at 0–4°C, precipitate material was collected by centrifuging at 10 000 *g* for 20 min and resuspended in PBS. The protein concentration in the 50% ammonium sulphate fractions was determined by the method of Lowry *et al.* (1951).

Expression of mycobacterial antigenic determinants in recombinant lysates was analysed by Western blotting using monoclonal antibodies CL1 (Mehra *et al.*, 1986), IIH9 and IIC3 (Gillis and Buchanan, 1982). Bacterial pellets and samples from the soluble and insoluble portions obtained after sonication of *E. coli* strains were mixed with sample buffer for SDS–polyacrylamide gel electrophoresis under reducing conditions (Laemmli, 1970) and incubated on a boiling water bath for 2 min. Samples (~5 μg of protein per lane) were applied to SDS–polyacrylamide gels (6% w/v, final acrylamide concentration) and subjected to electrophoresis at 15 mA per gel for 50 min using a mini-gel system supplied by Hoeffer Scientific Instruments (San Francisco, CA). Nitrocellulose blots were prepared from gels by electroblotting at 50 V for 1 h (Towbin *et al.*, 1979). Blots were washed with 0.2% Triton X-100 in PBS and non-specific binding was blocked by carrying out subsequent incubations in the presence of 5% dried milk powder in PBS–Triton. Development of Western blots with monoclonal antibodies and horseradish peroxidase conjugated secondary antibody was performed as described previously (Young *et al.*, 1986).

Synthetic peptides

The synthetic peptides were prepared using solid-phase methods on an Applied Biosystems 430 A peptide synthesiser as previously described (Townsend *et al.*, 1986). The particular regions of the protein that were synthesised were selected on the basis of the presence of a pattern of either four or five amino acids (charged/glycine followed by two or three hydrophobic residues and then a polar amino acid) (Rothbard, 1986). These two patterns have a high statistical correlation with known helper and cytotoxic T cell epitopes. The 65-kd protein contains 25 patterns of four amino acids and 12 patterns of five, which could be ordered by

analysing the amino acid sequence in comparison with previously defined T cell epitopes.

Preparation of lymphocytes

Mononuclear leucocytes reactive with *M. tuberculosis* were isolated from peripheral blood (PBMC) and the ascitic effusion of a patient by centrifugation on a discontinuous density gradient of Ficoll–Hypaque were resuspended in complete medium, RPMI 1640 supplemented with A+ serum, 2 mM L-glutamine and 100 IU/ml penicillin/streptomycin.

Isolation of human T lymphocyte clones

Human T cell clones reactive with *M. tuberculosis* were isolated as previously described (Lamb and Young, 1987). Briefly, ascitic lymphocytes (2.5×10^4/ml) were stimulated with MTSE (1 μg protein/ml) in 96-well, round-bottom microtitre plates (Nunc, Roskilde, Denmark) in complete medium. Lymphoblasts were enriched on Ficoll–Hypaque and cloned by limiting dilution (0.3 cells/well in Terasaki plates) in the presence of autologous irradiated (3000 rads) PBMC mixed (1:1) with ascitic lymphocytes (5×10^4/ml), MTSE (1 μg/ml) and 10% (v/v) interleukin-2 (IL-2; Lymphocult T, Biotest-Serum Institute GmbH, Frankfurt, FRG) (Lamb *et al.*, 1982). At day 7, growing clones were transferred to 96-well, flat-bottom microtitre plates and subsequently to 24-well plates. At each transfer the clones received filler cells, antigen and IL-2. The clones were expanded and maintained by the addition of fresh IL-2 every 3–4 days, and filler cells together with specific antigen every 7 days. Before use in proliferation assays the T cell clones were rested for 6–8 days after the last addition of filler cells.

Proliferation assays

Cloned T-lymphocytes (5×10^4/ml) were cultured with soluble antigen in the presence of autologous irradiated PBMC (1.25×10^5/ml) or EBV transformed B cells (5×10^4/ml) in a total volume of 200 μl in 96-well, round-bottom plates as previously described (Lamb and Young, 1987). After 72 h incubation [^{3}H]-methyl thymidine (1 μCi; [^{3}H]TdR; Amersham International, Amersham, UK) was added to the cultures for 8–16 h, and then harvested onto glass-fibre filters. Unfractionated lymphocyte cultures (1×10^6/ml) were pulsed with [^{3}H]TdR after 6 days incubation. Proliferation as correlated with [^{3}H]TdR incorporation was measured by liquid scintillation spectroscopy. The results are expressed as the mean counts per minute (c.p.m.) ± % error of the mean for triplicate cultures.

Acknowledgements

We thank Dr T.M.Buchanan for monoclonal antibodies II H9 and II C3; Dr B.R.Bloom for monoclonal antibody C1.1; Dr P.Kim for peptide 470–484; Dr T.M.Shinnick for preliminary communication of sequence for *M. tuberculosis* 65-kd protein; Drs M.Contreras and Dr.Mitchell for the blood samples. We are grateful to Professor J.Humphrey for his advice in the preparation of this manuscript.

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Received on February 17, 1987

Immunology 1987 60 1–5

A novel approach to the identification of T-cell epitopes in *Mycobacterium tuberculosis* using human T-lymphocyte clones

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Accepted for publication 15 September 1986

SUMMARY

Current approaches to the analysis of antigens involved in the cellular immune response to mycobacterial infection rely on the initial identification and isolation of molecular components using monoclonal antibodies. In order to overcome the constraints of this approach, we have utilized a procedure involving T-cell recognition of antigens fractionated by polyacrylamide gel electrophoresis (SDS–PAGE) and added to proliferation assays after blotting onto nitrocellulose membranes. Analysis of human T-cell responses to *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG by this procedure revealed distinctive patterns of reactivity to different molecular weight components indicative of the selective recognition of immunodominant and species-specific determinants. Human T-cell clones were subsequently derived, and SDS-PAGE immunoblotting was used to identify the antigen recognized by each clone. Three epitopes defined by individual T-cell clones were identified on separate polypeptides with molecular weights 16,000–18,000 (clone P53), 18,000–20,000 (clone P57) and 52,000–55,000 (clone P35). This study demonstrates the potential application of T-cell cloning in conjunction with SDS-PAGE immunoblotting for the dissection and analysis of the cellular immune response to pathogenic agents during human infection.

INTRODUCTION

A considerable effort has been made over many years to identify molecular components of pathogenic mycobacteria that are involved in stimulation or suppression of the human immune response during infection (Brennan, 1984; Daniel, 1984). The potential rewards of such research are the development of novel diagnostic reagents and the production of vaccines effective in controlling diseases such as leprosy and tuberculosis (Arnon, 1984). The generation and characterization of mouse monoclonal antibodies directed towards mycobacterial antigens have represented a significant advance in this area of research (Engers, 1985, 1986; Ivanyi, Morris & Keen, 1985) and, as a result of these studies, a limited number of protein antigens of *Mycobacterium tuberculosis* and *Mycobacterium leprae* have been identified. The independent generation of many monoclonal antibodies with overlapping specificities in separate laboratories suggests that these proteins represent immunodominant mycobacterial antigens with respect to the murine antibody response (Engers, 1985, 1986). Representative monoclonal antibodies from workshops organized by the World Health Organisation (Engers, 1985, 1986) have been used with considerable success to screen for expression of these major antigens in recombinant DNA libraries (Thole *et al.*, 1985; Young *et al.*, 1985a, b). Since protection from mycobacterial infection is a function of the cell-mediated immune system (Hahn & Kaufmann, 1981), several laboratories have initiated studies involving characterization of the human T-cell response to these antigens (Mustafa *et al.*, 1986; Ottenhoff *et al.*, 1986; Emmrich *et al.*, 1986; Young *et al.*, 1986).

While it seems likely that the monoclonal antibody-defined proteins are immunodominant for murine B cells (Engers, 1985, 1986; Ivanyi *et al.*, 1985), it is not clear that the same pattern of immunodominance will be reflected in the human T-cell response to mycobacteria. It is possible, for example, that antigens that cause marked T-cell proliferation during infection may stimulate little or no antibody formation following immunization with a mycobacterial extract. There is therefore a need to supplement the present monoclonal antibody-based approach to antigen identification with an approach that directly identifies antigens recognized by human T cells. The recent development of an SDS-PAGE assay for screening T-cell clones (Young & Lamb, 1986) provides the possibility of such an approach, and we describe here its application to the identification of antigens of *M. tuberculosis* involved in the human T-cell response.

Abbreviations: BCG, Bacillus calmette-guerin; [^{3}H]TdR, tritiated methyl thymidine; IL-2, interleukin-2; MBSE, *M. bovis* soluble extract; MTSE, *M. tuberculosis* soluble extract; SDS–PAGE, sodium dodecyl-sulphate–polyacrylamide gel electrophoresis.

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MATERIALS AND METHODS

Antigens

Mycobacterium tuberculosis H37Rv and *Mycobacterium bovis* BCG were grown for 8 weeks as a surface pellicle on Sauton's

medium. Soluble extracts were prepared by disruption of cobalt-irradiated organisms using a Braun MSK cell disintegrator at 4000 r.p.m. for 2 min at 5–10°. Bacterial debris was removed by centrifugation at 30.000 *g* for 1 hr. and supernatant material was filtered through a 0·45 micron filter. Protein concentrations in the soluble extracts were determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Preparation of immunoblots

Dot blots and SDS–PAGE immunoblots were prepared as described previously (Young & Lamb, 1986). Samples of mycobacterial extracts for electrophoresis contained 20 μg of protein per lane. Polyacrylamide gels were run on a 'mini-gel' apparatus (Hoeffer Scientific Instruments, San Francisco, CA) to a total length of 2 cm (Table 2) or 4 cm (Figs 1 and 2), or on a full-sized gel apparatus (BioRad Instruments, Richmond, CA) to a total length of 15 cm (Fig. 2, insert). Proteins were transferred to nitrocellulose by electroblotting as described elsewhere (Young & Lamb, 1986).

Preparation of lymphocytes

Mononuclear leucocytes isolated from peripheral blood (PBMC) and the ascitic effusion of a patient reactive with *M. tuberculosis* by centrifugation on a discontinuous density gradient of Ficoll-Hypaque (Boyum, 1968) were resuspended in complete medium, RPMI-1640 supplemented with 10% pooled A+ serum, 2 mM L-glutamine and 100 IU/ml penicillin/streptomycin.

Isolation of human T-lymphocyte lines and clones

Ascitic lymphocytes ($2{\cdot}5 \times 10^5$/ml) were stimulated for 6 days with a soluble extract of *Mycobacterium tuberculosis* strain H37Rv (MTSE; 1 μg protein/ml) in 96-well round-bottomed microtitre plates (Nunc, Roskilde, Denmark) in complete medium. The activated lymphocytes were isolated on Ficoll–Hypaque and expanded as a long-term T-cell line (P anti-Rv) with autologous irradiated (3000 rads) PBMC mixed (1 : 1) with ascitic lymphocytes (5×10^5/ml) and MTSE (1 μg/ml) in the presence of 10% interleukin-2 (IL-2; lymphocult T, Biotest-Serum Institut GmbH, Frankfurt, FRG) or cloned by limiting dilution as previously described (Lamb *et al.*, 1982). T-cell clones reactive with mycobacterial antigens were isolated as follows. Ascitic T lymphoblasts were diluted [0·3 cells/well in Terasaki plates (Nunc)] in the presence of irradiated autologous PBMC mixed (1 : 1) with ascitic lymphocytes (5×10^5/ml), 10% IL-2 and MTSE (1 μg/ml). Growing clones at Day 7 were transferred to 96-well flat-bottomed microtitre plates and subsequently to 24-well plates then 25 cm^2 flasks. At each transfer the clones received filler cells, antigen and IL-2. The clones were expanded and maintained by the addition of fresh IL-2 every 3–4 days, and filler cells together with specific antigen every 7 days. Before use in proliferation assays the T-cell clones were rested 6–8 days after the last addition of filler cells.

Proliferation assays

T-lymphocyte clones and long-term lines (5×10^4/ml) were cultured with soluble or nitrocellulose-bound antigen in the presence of autologous irradiated PBMC/ascitic lymphocytes (5×10^5/ml) in 96-well flat-bottomed microtitre plates as previously described (Young & Lamb 1986). Briefly, after 72 hr incubation the cultures were resuspended and the nitrocellulose strips discarded prior to the addition of tritiated methyl thymidine (1 μCi; [^{3}H-]TdR: Amersham International, Amersham, Bucks) for 8–16 hr and then harvested onto glass fibre filters. The duration of unfractionated lymphocyte cultures (1×10^6/ml) was 5 days. Proliferation as correlated with [^{3}H]-TdR incorporation was measured by liquid scintillation spectroscopy. The results are expressed as the mean counts per minute (c.p.m.) ± % standard error of the mean for triplicate cultures.

RESULTS

T-cell proliferation in response to nitrocellulose-bound antigen

T lymphocytes obtained from ascitic effusions or from the peripheral blood of tuberculosis patients were found to proliferate in response to *M. tuberculosis* extract supplied either as soluble antigen or in the form of a solid-phase antigen bound to nitrocellulose. Table 1 shows a representative experiment demonstrating proliferation of a T-cell line derived from an ascitic effusin. As described previously for the clonal respo a viral antigen (Young & Lamb, 1986), a higher concentration of solid-phase antigen was required to stimulate an equivalent response to that obtained with soluble antigen. No proliferation was observed in the absence of accessory cells, and the nitrocellulose itself was not mitogenic (Table 1).

Differential pattern of reactivity on the polyclonal T-cell response to SDS–PAGE immunoblots of *M. tuberculosis* and *M. bovis*

In order to identify the molecular weight of the components stimulating the proliferative response of the polyclonal T-cell population, mycobacterial extracts were subjected to SDS-PAGE and, following transfer to nitrocellulose membrane, the different antigen factions were tested in proliferation assays. Figure 1 shows the response of ascitic T cells to *M. tuberculosis* and *M. bovis* BCG extracts examined in this manner. A distinct

Table 1. Activated T lymphocytes recognize antigens blotted on nitrocellulose

			Response ([^{3}H]TdR incorporation c.p.m. ± SEM)	
T cells	APC	Antigen	Nitrocellulose bound	Soluble
+ *	−	—	375 ± 14	
+ †	+	—	180 ± 9	286 ± 23
+	+	MTSE 0·1 μg/ml	5482 ± 10	24.197 ± 21
+	+	+ 1·0 μg/ml	21.445 ± 16	30.997 ± 35
+	+	+ 10 μg/ml	31.620 ± 1	55.381 ± 20
+ ‡	−	+ 1·0 μg/ml + nitro	—	22.478 ± 18
+	−	IL-2	—	36.474 ± 36

H37Rv-reactive T-cell line (P anti-Rv 5×10^4/ml) was stimulated with MTSE as soluble antigen or nitrocellulose-bound as dot-blots together with irradiated autologous ascitic lymphocytes and PBMC (5×10^5/ml) as a source of antigen-presenting cells (APC). Proliferation as correlated with [^{3}H]TdR incorporation was determined at 72 hr. The results are expressed as the mean c.p.m. ± SEM of triplicate cultures. Control responses of T cells to (*) medium, (†) APCs without antigen, and (‡) soluble antigen in the presence of nitrocellulose.

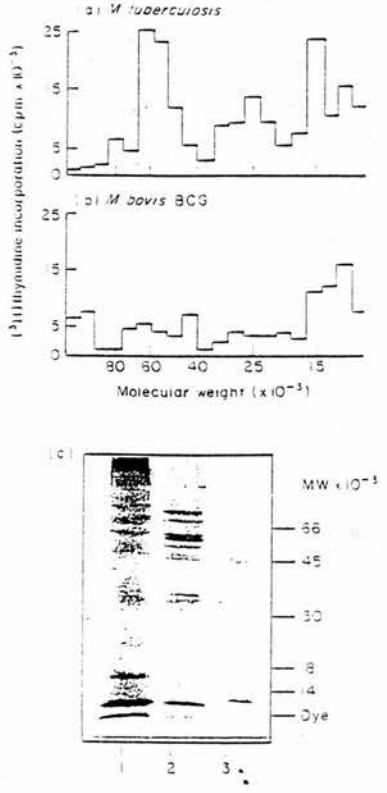

Figure 1. Differential pattern of reactivity of the polyclonal T-cell response to SDS-PAGE antigens on immunoblots of *M. tuberculosis* and *M. bovis* BCG. Unfractionated ascitic lymphocytes (1×10^6/ml) were cultured for 5 days with *M. tuberculosis* and *M. bovis* BCG on nitrocellulose blots (20 fractions) or as soluble antigen. Proliferation was determined as described in the legend to Table 1. The response to soluble *M. tuberculosis* and *M. bovis* BCG (1 μg protein/ml) was 112,411 ± 15 and 71,246 ± 9 c.p.m. ± SEM, respectively. Background proliferation of the T cells to nitrocellulose or to medium alone were 637 ± 14 and 587 ± 22 c.p.m. ± SEM. (a) *M. tuberculosis* and (b) *M. bovis* BCG extracts (20 μg protein per lane) were prepred and run on SDS-polyacrylamide gels, and proteins were blotted onto nitrocellulose: (c) A representative gel stained with Coomassie blue: Lane 1, *M. tuberculosis* extract; Lane 2, *M. bovis* BCG extract; Lane 3, molecular weight markers.

pattern of peaks and troughs in the proliferative response to *M. tuberculosis* extract was observed (Fig. 1a) with peaks particularly in the molecular weight regions of 51.000–66.000, 24.000–28.000, and 12.000–18.000. While the overall response of this T-cell population to *M. bovis* BCG extract at 1 μg/ml (71,246 ± 15) was comparable to the *M. tuberculosis* response (112,411 ± 15), quantitative and qualitative differences in response to the two extracts were observed during analysis by SDS-PAGE immunoblotting (Fig. 1a and b). The higher molecular weight fractions from *M. bovis* BCG (51.000–66.000 and 24.000–28.000) stimulated a lower response than those from *M. tuberculosis*, although comparable proliferation in response to the low molecular weight material (< 20.000) was observed. Visual inspection of polyacrylamide gels stained for total protein (Fig. 1c) did not indicate a marked difference in the concentration of proteins in these molecular weight regions between the two mycobacterial extracts.

Analysis by T-cell cloning

In order to carry out a more detailed analysis of the T-cell response to the antigens of different molecular weights, a set of T-cell clones was established from the T-cell population shown in Fig. 1. Nine of the clones were selected for detailed analysis and these are listed in Table 2.

All of the clones proliferated in response to the *M. tuberculosis* extract, while several showed little or no response to *M. bovis* BCG. Seven strongly proliferative clones (> 10.000 c.p.m. to *M. tuberculosis*-soluble extract) showed a clearly positive response to nitrocellulose-bound antigen, while the remaining two clones (P2 and P56) showed only a weak proliferation to solid-phase antigen. All of the clones were tested for recognition of high, medium or low molecular weight fractions obtained from 2-cm mini-immunoblots, and positive results (proliferation > 1000 c.p.m.) were observed with five of the clones (Table 2). Clones P35, P53 and P57 were then analysed using 4-cm SDS-PAGE immunoblots divided into 20 fractions each (Fig. 2). Clone P35 responded to two adjacent fractions in the high molecular weight region (50.000–60.000 (Fig. 2) and subsequent more detailed analysis using 70-fraction immunoblots indicated a molecular weight of 52.000–55.000 for the antigen recognized by this clone (Fig. 2a, insert). The other two clones (P53 and P57) that both showed some proliferation in response to *M. bovis* BCG recognized antigens with molecular weight 16.000–18.000 (Fig. 2b) and 18.000–20.000 (Fig. 2c), respectively.

DISCUSSION

The results presented in this paper demonstrate a novel and important approach to the molecular analysis of the antigens of a microbial pathogen that interact with the human cellular immune system. It is possible to use SDS–PAGE immunoblotting to analyse the contribution of antigens of different molecular weight to the overall polyclonal T-cell response to a mycobacterial extract. The extension of this analysis to screen the polyclonal response of tuberculosis patients and normal individuals to extracts prepared from different mycobacteria represents a potential approach to the identification of species-specific antigens and of antigens that are recognized as immunodominant during infection. Since the concentration of protein in each fraction of the gel has not at this stage been assayed, it cannot be stated whether the immunodominance represented by the peaks is a reflection of the concentration of particular antigens or of the concentration of T cells with appropriate specificities. Visual inspection of gels stained for total protein does not demonstrate the occurrence of particularly high concentrations of protein in sections of the gel stimulating maximum T-cell proliferation, and immunodominance based on immune recognition rather than on relative antigen concentration would therefore appear more likely. Occurrence of antigens causing a suppression of T-cell proliferation in the

Table 2. Specificity pattern of MTSE-induced T-cell clones for immunoblotted *M. tuberculosis*

	Clone no.								
	P2	P35	P48	P53	P56	P57	P65	P77	P83
(A) *Soluble antigen*									
1. MTSE	7251	28.115	11.584	45.507	8998	19.053	13.772	12.020	25.851
2. MBSE	818	406	1804	14.902	279	13.466	895	805	3155
3. IL-2 response	36.798	83.042	64.290	29.105	39.019	88.258	37.818	61.611	51.867
4. APC control	283	273	121	113	95	176	155	127	346
5. Medium control	86	109	105	75	55	176	74	103	83
(B) *Nitrocellulose-bound antigen*									
1. MTSE	1387	9195	4108	19.175	1441	9388	2804	7069	7868
2. Immunoblot > 50.000	221	5778	739	2039	380	575	167	351	479
3. Immunoblot 35.000–50.000	345	399	2580	4944	276	586	298	238	464
4. Immunoblot 20.000–35.000	533	430	231	699	126	464	353	252	497
5. Immunoblot 20.000	431	568	1387	7553	356	2431	884	538	1226
6. Nitrocellulose control	451	352	214	137	128	159	252	237	457

Cloned T cells ($2{\cdot}5 \times 10^4$/ml) were stimulated with MTSE or *M. bovis* BCG soluble extract. MBSE (1 μg protein/ml) as a soluble antigen (A1, A2) or the same concentration of MTSE bound to nitrocellulose (B1). Each clone was also tested with four sections prepared from 2-cm MTSE immunoblots corresponding to the approximate molecular weight ranges listed for samples B2–B5. Results from the major reactive section are underlined for each clone. Controls included medium alone (A5), with antigen-presenting cells (A4) and with uncoated nitrocellulose membrane (B6). Proliferation assays were performed as described in the legend to Table 1.

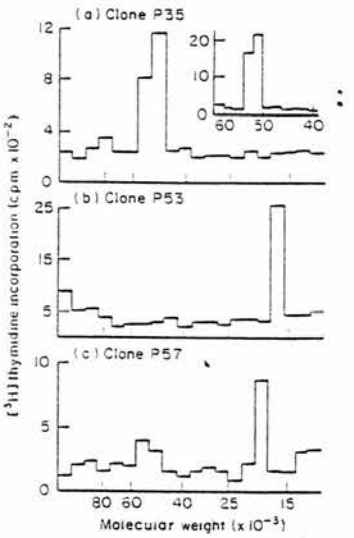

Figure 2. Identification of antigens recognized by *M. tuberculosis*-reactive T-cell clones using immunoblot analysis. T cells of clones P35, 53 and 57 ($2{\cdot}5 \times 10^4$/ml) were cultured with SDS–PAGE-separated immunoblots (20 fractions) together with irradiated PBMC/ascitic lymphocytes and assayed as described in the legend to Table 1. The specificity of clone P35 was analysed in more detail using the MW fractions (40.000–60.000) of a 15-cm (75 fractions) immunoblot [insert in (a)]. Results are shown as the mean of triplicate assays, the standard error for peak samples was less than 15% in each case.

areas of the gel corresponding to the troughs cannot be dismissed at this stage. It is also possible that the immunodominance apparent by this technique may reflect other properties of the proteins such as their degree of binding to nitrocellulose and the efficiency of processing and presenting particular antigens from the solid phase. While being aware of these reservations, analysis by SDS–PAGE immunoblotting does offer the potential of obtaining considerably more information than simple proliferation assays with unfractionated extracts, and this approach may be expected to lead to the identification of immunodominant antigens of important biological function of specificity in complex antigen mixtures.

In addition to the analysis of polyclonal responses, SDS-PAGE immunoblotting allows a new role for T-cell clones in the identification and analysis of mycobacterial antigens. Several authors have described the generation of human T-cell clones recognizing mycobacterial extracts (Mustafa *et al.*, 1986; Ottenhoff *et al.*, 1986; Emmrich *et al.*, 1986) and, in some cases, particular clones have been shown to respond to individual antigens identified using mouse monoclonal antibodies (Ottenhoff *et al.*, 1986), or by recombinant DNA techniques in association with the same antibodies (Mustafa *et al.*, 1986; Emmrich *et al.*, 1986). In this paper we have demonstrated a direct approach to the identification of the antigen specificity of human T-cell clones that is not dependent on an antibody-based analysis. Two of the T-cell clones described here (P53 and P57) recognize antigens in a molecular weight range 16.000–20.000 that overlaps with the 19.000 antigen recognized by known monoclonal antibodies (Engers, 1986) and further analysis to determine whether the T-cell antigen in either case is identical to the 19.000 protein will be of interest. A third clone (P35) recognizes an antigen in the molecular weight region 52.000–

55,000, which is well separated from the monoclonal antibody-defined proteins at 65,000 and 38,000 (Engers, 1986). This suggests that the T- and B-cell repertoires as regards their specificity for mycobacterial antigens need not be identical as has been reported for other antigens (Berzofsky, Richman & Kilon, 1979; Lamb & Green, 1983). It is interesting to note that this clone, which did not respond to *M. bovis* BCG, recognized an antigen in the molecular weight range that appeared to be relatively rich in *M. tuberculosis*-specific antigens. Of the 33 monoclonal antibodies analysed during the WHO workshop on antibodies to *M. tuberculosis*, none was found to discriminate completely between tubercle bacilli and *M. bovis* BCG (Engers, 1986). This study therefore not only demonstrates a novel approach to the analysis of antigen specificity of T-cell clones, but has also resulted in the identification of an *M. tuberculosis* antigen that is not represented in the previous set of monoclonal antibody-defined proteins. Not all of the T-cell clones investigated in these experiments recognized the SDS-PAGE immunoblots, and therefore this procedure may not be applicable for all T [illegible] subsets or for those T cells with low-affinity receptors. Furthermore, where T-cell clones responded to more than one fraction of the four fraction (2-cm) immunoblots, this may result from inadequate separation of the determinants, or it may be that one determinant can exist on multiple molecular weight proteins as evidence by monoclonal antibodies recognizing the 65,000 'multiple band' antigen of mycobacteria (Ivanyi *et al.*, 1985). Therefore, further analysis of the T-cell clones described in this paper using extracts from different strains of mycobacteria and using *Escherichia coli* clones expressing recombinant mycobacterial DNA (Young *et al.*, 1985a, b) is currently in progress.

Thus, the experiments described here indicate two potentially important areas for the application of SDS-PAGE immunoblotting to further research on the molecular analysis of mycobacterial immunity. These are the analysis of immunodominant antigens by screening of polyclonal responses, and the identification of novel antigens and epitopes using T-cell clones. A panel of well-characterized human T-cell clones recognizing different mycobacterial antigens could prove useful in complementing the set of antigens previously defined by mouse monoclonal antibodies (Engers, 1985, 1986). Screening of recombinant DNA libraries (Thole *et al.*, 1985; Young *et al.*, 1985a, b) with such T-cell clones could provide an approach towards the isolation of the relevant antigens in quantities suitable for thorough immunological analysis.

ACKNOWLEDGMENTS

Blood donors were generously provided by Dr M. Contreras from the National Blood Transfusion Centre, Edgware, and Dr D. Mitchell, University College Hospital, London. We also thank Drs J. Ivanyi, C. Moreno, A. Rees and E. Zanders for helpful discussions, and Mrs E. Hall for excellent secretarial assistance.

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0022-1767/89/1421O-3599$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 142, 3599–3604, No. 10, May 15, 1989
Printed in U.S.A.

MOLECULAR STUDY OF THE T CELL REPERTOIRE IN FAMILY CONTACTS AND PATIENTS WITH LEPROSY

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The specificity of lymphocyte proliferative responses of 22 family contacts and 7 patients with leprosy were analyzed using Ag fractions from soluble extracts of *Mycobacterium leprae* and *Mycobacterium tuberculosis*. Fractions 10–100 kDa m.w. from each extract were separated by SDS-polyacrylamide gel electrophoresis, electroblotted to nitrocellulose membrane and solubilized for use in lymphocyte culture. The main immunogenic fractions for both contacts and patients had m.w. of 12,000 to 22,000, 35,000 to 40,000, and 65,000. Determinants which were either distinct or shared by the two extracts were active in each of the immunogenic fractions. Lymphocyte proliferation following stimulation with separated Ag was found also in five subjects who failed to respond to the whole soluble extracts. Stimulatory synthetic peptides were identified for the 65 kDa protein Ag. This technique has permitted the screening of the T cell immune repertoire for the identification of the immunodominant Ag which merit further purification and molecular characterization.

It is generally believed that the nature of T cell mediated immune responses to *Mycobacterium leprae* determine whether the infection will be self-limiting or pathogenic to the host. Moreover, the clinical spectrum from the tuberculoid to the lepromatous pole is represented by a change from pronounced reactions to T cell anergy in response to *M. leprae* Ag. This information is based on delayed type hypersensitivity and in vitro lymphocyte proliferation to crude soluble extracts from *M. leprae* (1–3). The role of individual Ag in protective or pathogenic host interactions and hence the spectrum of the disease is not yet understood. Earlier studies which reported anergy to *M. leprae* but responsiveness to tuberculin stimulated the interest in *M. leprae*-specific constituents with putative suppressor function for the response to the common mycobacterial Ag (4–6). Cloned T cells of defined specificity have been described (7, 8), but without knowledge about their relative representation in the immune repertoire in relation to *M. leprae* infection or disease. Nevertheless, functional studies ascribed putative suppressor activity to certain T cell clones (9, 10).

This study analyzes the specificity of polyclonal T cell proliferation responses of leprosy contacts and patients. Ag contained in soluble extracts of *M. leprae* and *M. tuberculosis* were separated on the basis of m.w. within the range of 10 to 100 kDa by SDS-PAGE and were used, after electroblotting and solubilization for lymphocyte stimulation in vitro (11, 12). The specific aims of the study were: a) to investigate the most frequent immunogenic fractions; b) to compare the responses of patients with family contacts; and c) to find out if separated fractions are able to stimulate responses of lymphocytes which fail to proliferate in the presence of whole soluble extracts. Parallel analysis was performed on the specificity of serum antibodies.

MATERIALS AND METHODS

Patients and contacts. Blood in citrate-dextrose was obtained from seven patients with leprosy and 22 of their family contacts, informed consent being obtained from themselves or their parents/guardians. The disease was classified according to the criteria of Ridley and Jopling (13). Patients (3 male, 4 female) aged between 31 to 60 years had been taking anti-mycobacterial chemotherapy for 4 to 17 years. All patients had lived for many years in leprosy-endemic areas of the Indian Subcontinent except for patient C3-c who originated in the Yemen. None of the 12 contacts aged 17 years or less had spent more than a few weeks in leprosy-endemic areas but all 8 older contacts had lived in endemic areas for a number of years and could have been exposed to *M. leprae* from other infectious cases.

Mycobacterial extracts. MLSE, a filtered sonicate of *M. leprae* obtained from infected armadillo livers (batch CD52), was a gift from Dr. R. J. W. Rees, National Institute for Medical Research, London, U.K. *M. tuberculosis* strain H37Rv bacilli grown on Sauton's medium and killed by 2.5 Mrad from a ^{59}Co source were disrupted in a Braun MSK cell homogenizer and the MTSE, batch 5, was obtained by centrifugation for 60 min at 47,000 × *g*.

Ag fractions. MLSE or MTSE (50 or 100 μg per gel) were applied, using a blank comb with a single reference well for m.w. markers (Sigma Chemical Co., Poole, Dorset, U.K.). Protein bands were separated using SDS-PAGE (12% w/v acrylamide in the running gel and 4.8% in the stacking gel) under reducing conditions at 15 mA per gel for 50 min in a Minigel electrophoresis system (SE 250 Hoeffer Scientific Instruments, San Francisco, CA). Both mycobacterial extracts contained several protein constituents, apparently following staining with Coomassie blue.

The proteins were electrophoretically transferred to nitrocellulose paper in the TE22 Apparatus (Hoeffer Scientific Instruments, San Francisco, CA) using 0.02 M Tris-glycine buffer (pH 8.3) containing 20% methanol for 1 h at 50V. Nitrocellulose membranes (7.5 mm × 40 mm) were cut into 20 horizontal sections each 2 mm wide, corresponding to 12 to 92 kDa m.w. fractions. Strips were then solubilized as described previously (12) by incubation and intermit-

Received for publication October 21, 1988.
Accepted for publication February 10, 1989.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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tent mixing for 1 h with 500 µl DMSO and nitrocellulose particles were precipitated by the addition of 500 µl sterile 0.05 M carbonate-bicarbonate buffer while vortexing the mixture vigorously. The nitrocellulose particles were suspended in 0.5 ml of RPMI 1640 (Flow Laboratories, Rickmansworth, Herts.) after removal of DMSO by repeated washing and centrifugation with PBS. The emulsified fractions from 16 replicate nitrocellulose membranes were pooled, and aliquoted at −20°C for use in proliferation assay. M.w. were assigned to fractions using markers (Sigma) which were stained following separation with amido black.

Lymphocyte proliferation assays. PBMC were isolated on a Ficoll-Hypaque density gradient and stored in liquid N_2. Thawed cells were suspended (10^5 or 5×10^4 ml) in 96-well microtitre trays in RPMI-1640 supplemented with 2 mM L-glutamine, 100 IU of penicillin, 100 µg of streptomycin per ml and 10% human A^+ serum: 20 µl of the 20 separate fractions were added to each well at the initiation of these cell cultures. After seven days of incubation, the cultures were pulsed with 1.0 µ Ci of tritiated methyl thymidine (^{3}HTdR: Amersham International; sp. act., 25 Ci/mmol) for 8 to 16 h and harvested onto glass-fibre filters. Proliferation, as correlated with ^{3}HTdR-incorporation, was measured by liquid scintillation spectroscopy. Mean counts per minute ± standard error of the mean for triplicate Ag-containing cultures were converted to stimulation indices (SI) in relation to the saline control culture.

Antibody assay. Nitrocellulose membranes prepared by blotting of SDS-PAGE separated fractions of MLSE or MTSE were cut vertically into 3 mm strips, and were washed with 0.1% Triton X-100 in PBS. Non-specific binding was blocked by incubation with 5% reconstituted dried skimmed milk in PBS-Triton. Strips were incubated with sera diluted 1:100 in PBS-Triton for 1 h at 20°C with shaking. After washing with Triton X-100 in PBS peroxidase-labelled affinity-purified goat anti-human IgG (gamma chain) or anti-human IgA (alpha chain) (Sigma) diluted 1:1000 in PBS-Triton was added to the individual nitrocellulose strips and incubated for 1 h at 20°C. After color development (5 min) using diaminobenzidine dihydrochloride (0.1 mg/ml, Sigma) in 0.1 M citrate buffer (pH 5) with 0.01% H_2O_2, strips were washed and dried.

RESULTS

The functional viability of the lymphocytes following frozen storage/thawing was evaluated by their PHA responsiveness. Satisfactory responses with mean 22.8 ± 2.52×10^{-3} cpm were obtained. Background proliferation in the presence of emulsified nitrocellulose without Ag varied from 200 to 2000 cpm. All lymphocytes were tested with fractions prepared from gels loaded with either 50 or 100 µg soluble extract. The results obtained at the two Ag doses were in most instances comparable both in the pattern and magnitude of the proliferation. A representative example of this is demonstrated in Figure 1. Responses to individual fractions, corrected for the corresponding background control, were considered positive when thymidine uptake was fivefold and strongly positive when it was tenfold the control. The results from all tested patients and contacts evaluated on this basis are shown in Figure 2. The obtained patterns of responses to fractions derived from SDS-PAGE fractionated MLSE (left column) and MTSE (right column) indicate that almost all stimulatory activities could be classified into three regions: 65 kDa, 35 to 40 kDa and a broader 12 to 22 kDa region. The latter low m.w. fractions clearly contained the most frequently immunogenic Ag; however, polypeptide fragments from larger native molecules may have partly contributed to this activity. The immunodominance of the separated fractions showed a similar pattern for contacts and patients.

The role of species-specific and cross-reactive determinants from each m.w. fraction can be at least partly ascertained by the comparison between MLSE and MTSE-derived fractions (Table I). The results suggested that both species-restricted epitopes and those cross-reactive between *M. leprae* and *M. tuberculosis* were

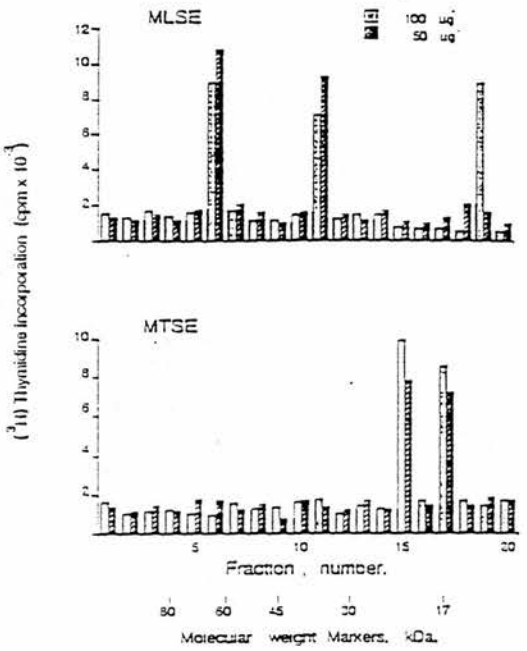

Figure 1. Reproducibility of lymphocyte stimulation patterns at two doses of Ag. Responses of one donor (C9-s) to concurrently separated Ag fractions.

immunogenic and contributed in about equal proportions when comparing the individual responders. This was observed with the 65, 35 to 40 as well as the <22 kDa fractions. Although the response patterns showed considerable individual differences, the frequency of responders to the three main immunogenic regions was similar when comparing leprosy patients and contacts.

All lymphocyte samples were also examined for proliferative responses to the whole soluble extracts, MLSE and MTSE. Two patients and five contacts failed to give a pronounced (SI > 5) proliferation response to the mixture of Ag contained in both extracts. While two of these contacts (LL-i and BL-ii source case) also failed to respond to the Ag fractions, lymphocytes from the other three contacts and two patients did respond significantly to at least one of the <22 kDa fractions: four of these donors responded also to 65 kDa and two donors to the 35 kDa fraction. Representative patterns showing the amplified proliferation in cultures with fractions when compared with whole soluble extract are demonstrated in Figure 3.

It was of interest to examine the possibility that Ag when presented from nitrocellulose are more stimulatory when compared with the native soluble extracts. Soluble mycobacterial extracts were incubated in sample buffer and polyacrylamide reagents, electroblotted onto nitrocellulose and prepared as emulsified particles under conditions corresponding with the technique used for the electrophoretically separated fractions. Graded amounts of processed and soluble preparations were then tested for their stimulatory potency in culture, using the PMBC

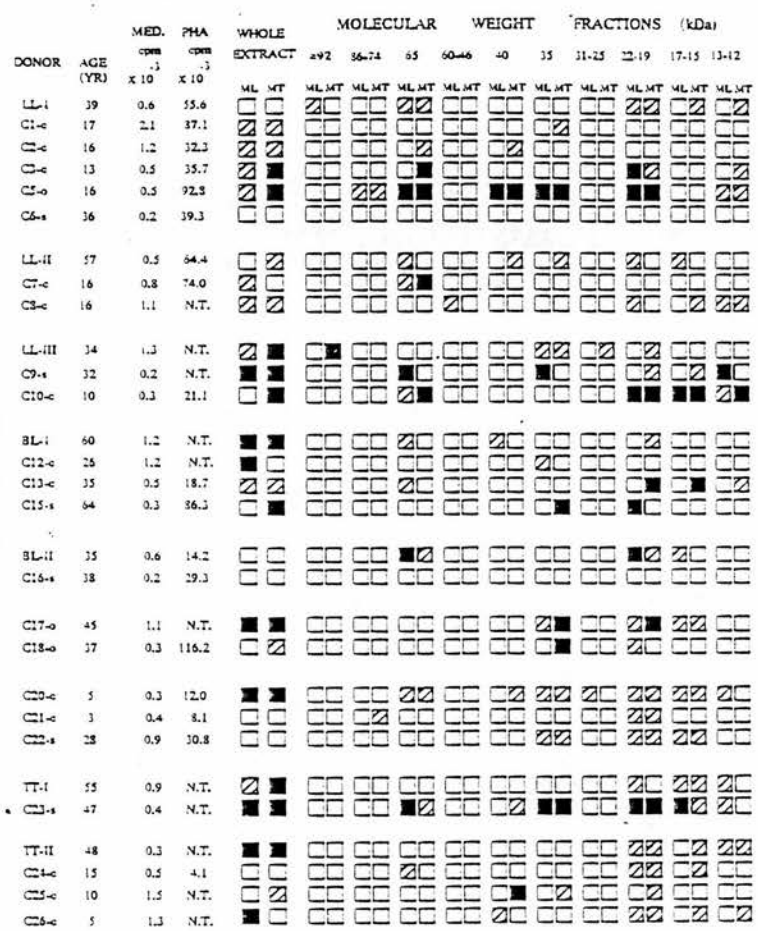

Figure 2. The pattern of proliferative responses to fractionated soluble extracts. Donors: patients (LL, BL, or TT) are listed with their respective contact (C) children (c), spouse (s) or other (o) relatives. Fractions were derived from SDS-PAGE-separated MLSE (ML) or MTSE (MT) applied to SDS-PAGE at the 100 μg/ml concentration. Counts of [^{3}H]TdR uptake are given for saline and PHA (10 μg/ml)-containing cultures. Whole soluble extracts (MLSE, MTSE) were added at 100 μg per ml culture. ■ = SI > 10, ▣ = SI 5–10; □ SI < 5. Source case (BT) lymphocytes for contacts C17–18 and C20–23 were not tested.

TABLE I

Frequency of proliferative and antibody responses to SDS-PAGE fractions

Assay	Ag fraction M_r (kDa)	Number of Responders[a]					
		Contacts (22)			Patients (7)		
		MLSE only	MTSE only	MLSE plus MTSE	MLSE only	MTSE only	MLSE plus MTSE
Proliferation	65	3	2	5	2	0	2
	40–30	3	6	6	0	1	1
	22–19	3	3	10	2	2	3
	17–15	0	5	5	2	2	1
	13–12	3	3	3	1	1	1
	whole extract	3	4	10	1	1	3
Antibody	>92	3	0	0	4	0	0
	70	0	6	0	0	1	0
	65–55	0	12	0	0	4	0
	40–30	6	2	5	2	0	3
	14	0	3	0	0	3	0

[a] Proliferation: SI > 5; Antibody: bands demonstrable by immunoblotting.

from a patient with tuberculoid leprosy (Fig. 4). The results showed an approximately twofold amplification of proliferation with the tested two lower doses of processed MTSE whereas the responses to the two tested MLSE preparations were equivalent.

PBMC from contacts C9-s, C10-c, C23-s, C13-c and C7-c all reactive with the recombinant 65 kDa Ag were analyzed for their ability to recognize synthetic peptides

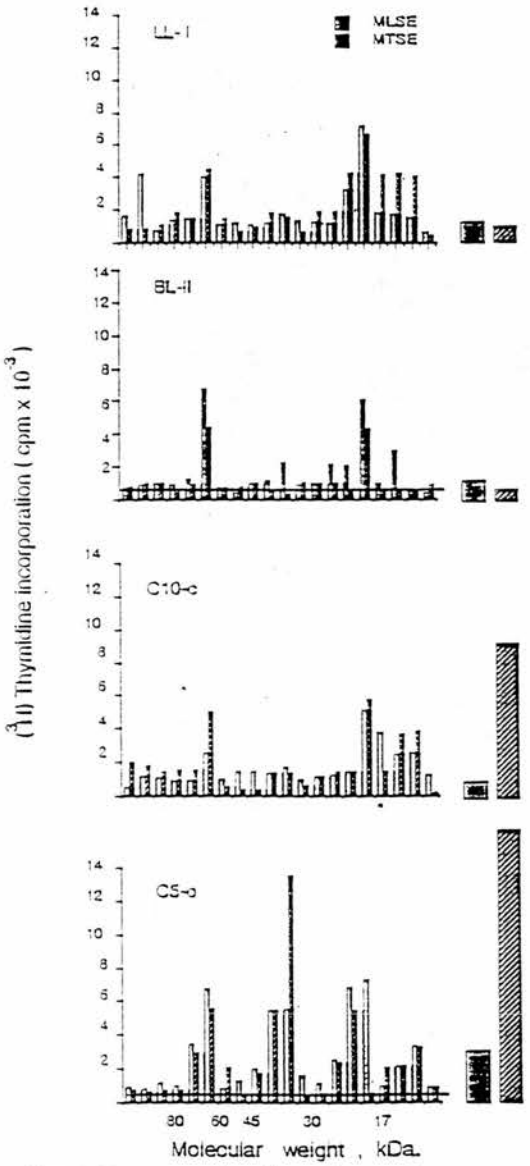

Figure 3. Demonstration of amplified proliferation with separated antigens compared to whole soluble extracts. Lymphocyte donors: see figure 2.

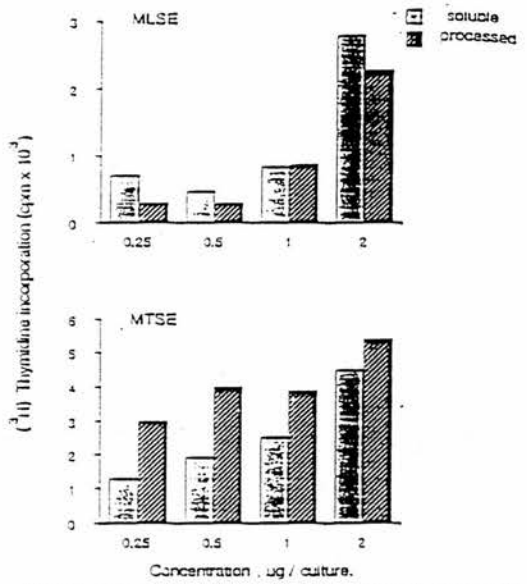

Figure 4. Comparison of proliferative responses to processed and soluble mycobacterial extracts. Each extract (100 µg) was processed under conditions corresponding to the preparation of SDS-PAGE fractions. Mean proliferative responses of PBMC from triplicate wells are presented.

derived from the sequence of *M. leprae* 65 kDa protein (14). Of the five subjects tested only three responded to the peptides and in each individual the pattern of recognition was different (Table II). Donor C9-s responded to peptides corresponding to the sequences 65 to 85, 153 to 171 and 195 to 219. This latter sequence was recognized by PBMC from C10-c and C23-s that also responded to residues 390 to 412 and 65 to 85 respectively. None of the individuals tested responded to sequence 180 to 196.

The specificity of serum antibodies from all lymphocyte donors was determined by the immunoblot technique. The m.w. of demonstrable bands on MLSE and MTSE blots are presented in Table I. The majority of the tested sera reacted with MTSE and fewer reactions were detected with MLSE. There was no correlation in the m.w. of the antibody binding and T cell stimulatory fractions when comparing individual contacts and patients. It is of particular interest that six contacts showed binding to a 35 kDa band with specificity for *M. leprae*. However, additional testing by competition assay (16) using the ML04 mAb (anti-35 kDa) showed positive values only in LL and BL patients, thus confirming the selective immunodominance of this epitope for multibacillary leprosy (results not shown).

DISCUSSION

In view of the complexity of the T cell proliferative repertoire in leprosy, we placed emphasis on the analysis of the responsiveness of healthy family contacts, hoping that their responses might be limited to fewer Ag. This study also had the advantage of being carried out in a non-endemic country (UK) where the potential source of infection was a single household member and there was relatively limited stimulation by environmental mycobacteria. For the analysis of the specificity of the T repertoire, SDS-PAGE fractionated Ag (MTSE and MLSE) was transferred to nitrocellulose and added to T cell proliferation assays as an emulsion (12). This technique has the

TABLE II

T cell recognition of peptides from the 65 kDa protein of mycobacteria

DONOR	medium	65 kDa*	65–85*	116–137	153–171	180–196	195–219	390–412
C9-s	0.2*	5.2	10.7	0.4	13.4	0.5	10.5	0.8
C10-c	0.3	3.3	0.5	0.7	0.3	0.5	4.3	3.7
C23-s	0.4	5.7	4.6	2.5	0.7	0.9	1.6	0.7
C13-c	0.5	5.1	0.3	0.2	0.3	0.5	1.4	0.2
C7-c	0.8	3.9	0.4	0.5	0.7	0.7	0.6	0.7

* Recombinant 65 kDa protein from *E. coli* (21).

* Peptides synthesized from the sequence of M. leprae 65 kDa protein (22). Ag were added to the cultures at 1 or 10 μg/ml and the highest proliferation induced with either concentration is shown.

* Results are expressed as mean cpm × 10^{-3} of triplicate cultures with SEM <20%. Responses >5-fold over background are underlined.

advantages that extensive biochemical purification of individual Ag components is not required and that there is no pre-selection of Ag based on other forms of immunodetection such as serology. The results demonstrated clear resolution between Ag when separated into 20 fractions of 12 to 92 kDa m.w. The main technical advance, which considerably helped the standardization of the analysis, was the pooling of fractions from replicate runs into batches which provided identical stimulants for as many as 400 lymphocyte cultures.

Recent results of lymphocyte proliferative patterns to SDS-PAGE separated fractions in healthy staff with extensive occupational contact with leprosy patients showed most frequent responsiveness to low m.w. material in the region of 11 to 16 kDa from *Mycobacterium bovis* and 22 to 26 kDa from *M. leprae* (17). Evaluation of responses in our study showed three main proliferation-inducing fractions with 19 to 22, 35 to 40 and 65 kDa m.w. Individual donor lymphocytes proliferated with fractions from *M. leprae* or *M. tuberculosis* alone or with corresponding fractions from both soluble extracts. This could reflect the immunogenicity of both common and distinct epitopes in the genetically polymorphic human population, although such a conclusion is limited by the possibility that homologous proteins might be of slightly different m.w. Polypeptides resulting from partial degradation of native molecules could have contributed to the marked stimulatory activity in the <22 kDa m.w. fractions observed. However, the paucity of stimulation by fractions in 40 to 65 kDa region was surprising considering that the 65 kDa protein is particularly susceptible to enzymatic proteolysis (18). It is possible that the fragmentation of the 65 kDa protein had disrupted or modified sites involved either in T cell recognition or distinct functional sites that may influence selective processing of the protein. Alternatively, non-specific inhibitors of T cell proliferation co-migrating in that region could have overridden the stimulatory effects. Previous studies have demonstrated that lipoarabinomannan which co-migrates at a similar m.w. is able to inhibit non-specifically T cell proliferative responses to protein Ag (19).

The patterns of proliferation found in this study showed a considerable degree of individual variation, but without a significant difference in pattern between contacts and patients (Table I). Overall, the patterns of Ag reactivity observed within and between the families may reflect in part the polymorphism in the MHC genes of the individuals investigated. It would be of particular interest to know if there are any associations between the form of leprosy or family relationship with the Ag specificity. It appeared that responses to the 65 kDa fractions prevailed in both case and contacts of families with a lepromatous (11/18) rather than tuberculoid (3/11) source case. In contrast, responses to the 22-19 and 17-15 kDa fractions were more frequent in tuberculoid than in lepromatous families. However, these apparent associations on the present limited number of examined cases did not attain statistical significance. It will be of interest to ascertain whether any of the observed differences are attributable to lymphocyte priming or to differences in Ag presentation by the respective accessory cells. Furthermore, the role of donor HLA haplotype which is known to control certain responses to mycobacterial Ag (20) would need to be evaluated.

The finding of proliferation in response to individual Ag in five donors who failed to respond to the whole soluble sonicate is of considerable interest. One possible explanation for this could be that SDS-PAGE/nitrocellulose/DMSO-processed Ag have enhanced stimulatory potency compared with soluble Ag. This interpretation was indicated by the previously reported 20 to 80 fold enhancement of responses by separated particles (12), although in comparable experiments in this paper only a modest enhancement of proliferation was observed with particulate MTSE and none with MLSE. Whether enhancement or inhibition of the response to unfractionated Ag presented in particulate form is observed for a particular individual will depend on the summation of the different effects for the various Ag components and MHC haplotype of the individual. However, the procedure can lead also to loss of antigenic activity, at least for certain Ag (21). Therefore, an alternative interpretation may be the abolition of a "suppressor" constituent by the technical processing or separation of the soluble extract. The latter mechanism, which had been considered in other studies (4, 9, 10, 17) would need to be examined by more detailed investigation.

Previous serological studies have consistently shown elevated antibody levels in multibacillary forms of leprosy only (22). Antibodies to the ML04 epitope of the 35 kDa protein were previously detected by competition assay in only a few (4%) of leprosy contacts (23) but in the majority of volunteers vaccinated with 5×10^8 killed *M. leprae* (24). In this study, several of the contact sera reacted with a 35 kDa band of *M. leprae*: since these sera were negative by the ML04-competition assay, the data suggest that antibodies were directed to additional epitopes on the 35 kDa molecule. Immunoblotting demonstrated binding to a band >92 kDa of *M. leprae* which may correspond to the 98 kDa band noted by other investigators (17) and as yet not identified by mAb.

Since T cells specific for the 65 kDa protein appear to represent a major component of the T cell repertoire reactive with mycobacteria, the fine specificity was in-

vestigated using synthetic peptides derived from this protein. Selection of these peptides was based on the presence of sequences similar to those identified in previously defined T cell epitopes (14). Since it is likely that each peptide may contain more than a single T cell epitope and that Class II MHC proteins are able to bind a variety of peptides (25) it was not surprising that some individuals, C9-s and C23-s recognized multiple peptides. Furthermore, the failure of the T cells from two of the individuals to respond to any of the six peptides tested, although reactive with the recombinant protein suggests that as yet other undefined epitopes are present in the 65 kDa protein.

The presented results illustrate the complexity of T cell Ag recognition but also represent a lead for the identification of immunodominant epitopes at the population level. The fractionated Ag represents a distinct advance over the use of whole soluble extract from *M. leprae* for the analysis of the T cell repertoire in *M. leprae* infected subjects. It is apparent however, that the physical form of presentation may be important and could have distinct characteristics for each of the relevant Ag. Antibody and T cell proliferation patterns did not overlap at the level of the individual, indicating that other than helper function is exercised by the T cells. Therefore, additional assays for lymphokine secretion combined with the Ag specificity will be of interest in future studies.

Acknowledgments. We thank J. Rothbard (Imperial Cancer Research Fund) for peptides, J. van Embden (National Institute of Public Health, Bilthoven) for the recombinant 65-kDa protein, and R. Lathigra for help with computer graphics.

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The 65kDa antigen of mycobacteria – a common bacterial protein?

The 65 kilodalton antigen of Mycobacterium tuberculosis *and* M. leprae *is a well-characterized, strongly immunogenic protein eliciting antibody and T-cell responses in infected patients. Recent studies have disclosed regions of cross-reactivity between the 65kDa antigen and proteins in many other bacterial species. These include the product of the ams gene in* E. coli *which is involved in the processing of RNA. Here Douglas Young and his colleagues discuss these observations, the significance of the 65kDa antigen and its possible role in the pathogenesis of mycobacterial and other diseases.*

Douglas B. Young, Juraj Ivanyi, Josephine H. Cox and Jonathan R. Lamb

The challenge of understanding the mechanisms underlying the immune response to mycobacteria has been taken up by successive generations of scientists and renewed efforts are currently underway to use improved methods for analysis of mycobacterial antigens and for isolation and characterization of T cells. The effective immune response is thought to involve recognition of mycobacterial antigens by helper T lymphocytes, followed by activation of macrophages and perhaps stimulation of cytotoxic T cells. A key area of research is therefore to identify mycobacterial antigens which are involved in recognition by T cells.

A major advance in the identification of mycobacterial protein antigens was accomplished by monoclonal antibodies directed to components from *Mycobacterium tuberculosis* and *Mycobacterium leprae*[1]. One of the antigens which was identified by a high proportion of monoclonal antibodies – particularly those raised against *M. leprae* – has been generally referred to as the '65kDa antigen'. Monoclonal antibodies directed to this antigen frequently bind to bands with multiple molecular weights during western blotting of extracts from *M. tuberculosis* and *M. leprae*, although this banding pattern seems to be less marked in the case of more rapidly growing mycobacteria[2]. The most likely origin of the multiple banding is that an original protein of about 65 kDa undergoes progressive proteolytic degradation to multiple fragments each containing a subset of the total antibody binding sites of the full length molecule.

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Monoclonal antibodies recognizing epitopes situated towards the N terminus of the molecule (for example, Y1.2 – see Fig. 2) show very limited recognition of low molecular weight bands and this would be consistent with an initial site of proteolysis being located near to the N terminus. Antibodies binding in the C terminal region (e.g. IIIC8) do show extensive 'multi-banding'.

The 65kDa antigen has been described as 'cell-wall-associated'[2] since much of the antigen is found in the insoluble fraction remaining after disruption of the mycobacterial cells. Other authors have proposed a 'periplasmic' location for this antigen and have demonstrated release of the molecule into culture supernatants during growth of *Mycobacterium bovis* under conditions of zinc deficiency[3]. Since the amino acid sequence of the protein (see below) contains no strikingly hydrophobic regions, a close association of such a molecule with the lipid-rich cell wall of mycobacteria seems unlikely. While immuno-electron microscopy may be expected to provide definitive information on the location of the 65kDa antigen, an interaction with ribosomes would be consistent with some of the data discussed below.

Cloning of the 65kDa antigen

Most of the mycobacterial antigens recognized by monoclonal antibodies have been expressed from *M. leprae* and *M. tuberculosis* genomic libraries using the λgt11 system[4,5]. Recombinant clones expressing all, or portions, of the 65kDa antigen were found at a particularly high frequency. It has been shown that the gene for the 65kDa protein can be expressed in *E. coli* regardless of its orientation with respect to promoters on the cloning vector, suggesting that, in contrast with some other mycobacterial enzymes[6], the mycobacterial promoter for this gene can be recognized by *E. coli*[7–9]. The proteins from *M. leprae* and *M. tuberculosis* contain a striking degree of sequence homology, differing in only 26 amino acid substitutions[8,9]. From the primary sequence it can be predicted that the 65kDa antigen contains extensive α-helical secondary structure with no marked hydrophobic regions and no cysteine residues.

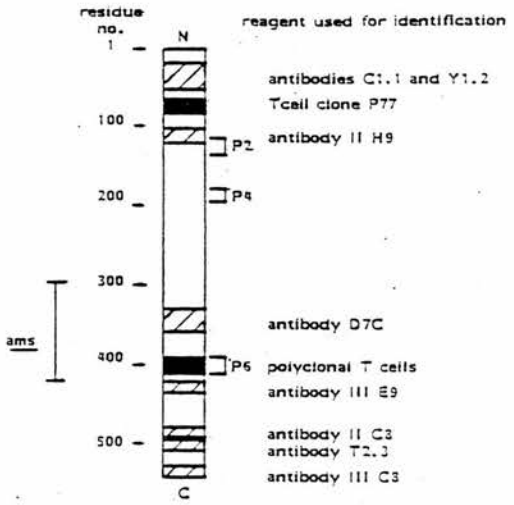

Fig. 1. Location of antigenic determinants on the 65kDa protein.

The 65kDa antigen of M. leprae is shown with amino acids numbered from the alanine which has been shown to be the N terminal residue of the protein in mycobacteria[3]. The position of the antibody binding sites (hatched boxes) was determined by sublibrary mapping[4]. T-cell determinants (solid boxes) were identified by sublibrary mapping and predictive sequence analysis[14]. Areas corresponding to synthetic peptides containing predicted T-cell determinants (P2, P4 and P6) are also shown[14]. Overlap with the ams gene product[17] is indicated on the left of the figure.

Mapping of antibody epitopes

Competitive binding assays showed that monoclonal antibodies recognize at least 14 spatially separate epitopes on the 65kDa protein[10]. Most of the monoclonal antibodies bind to the 65kDa antigens from both *M. leprae* and *M. tuberculosis* but antibodies IIIE9 and IVD8 recognize an epitope which is unique to the leprosy bacillus[11], while TB78 recognizes an epitope present only in *M. tuberculosis* and *M. bovis*[12]. By screening a DNA sublibrary prepared by cloning fragments of the 65kDa gene in λgt11, it was possible to determine the amino acid sequence which constitutes the epitope for six of the monoclonal antibodies – including the *M. leprae*-specific antibody IIIE9 (Ref. 8) (Fig. 1).

The complete success of the sublibrary mapping approach was unexpected, in that one might have predicted that many of the antibodies would be directed to non-linear determinants and would therefore fail to bind to the antigen fragments. The observed high frequency of monoclonal antibodies directed to linear determinants on the 65kDa antigen could be the result of the fragmented nature of the antigen preparations used for immunization. Alternatively, the fact that many of the investigators used western blot analysis for characterization of the monoclonal antibodies may have favoured selection of antibodies able to bind to denatured antigen. A third possible explanation would be that some structural feature of the 65kDa protein particularly promotes binding of antibodies to linear determinants. This might be the case, for example, if the molecule exists in a predominantly unfolded configuration with a high proportion of surface-exposed residues. Such a structure might also be consistent with the enhanced sensitivity of this antigen to attack by proteolytic enzymes. Preliminary analysis of sera from tuberculosis patients by sublibrary mapping suggests that non-linear epitopes may be important in the polyclonal antibody response to the 65kDa protein.

Mapping of T-cell epitopes

Several groups have reported the isolation of human T-cell clones reactive with the 65kDa antigen[13–15] and the purified protein elicits a strong delayed-type hypersensitivity response in immunized guinea pigs[3]. Two of the epitopes recognized by human T lymphocytes have been mapped by combining λgt11 sublibrary screening with a second approach which involved analysis of the 65kDa antigen for the presence of sequence motifs with similarity to previously defined T-cell epitopes[14]. Based on this analysis, six peptides were synthesized and two were shown to contain T-cell epitopes (Fig. 1). While these two epitopes were recognized by the individual used in this particular study, more T-cell epitopes may be found on the 65kDa antigen once the analysis is broadened to include individuals with differing HLA types.

Cross-reactivity with *E. coli*

When extracts from clones containing recombinant DNA were screened on western blots, some of the monoclonal antibodies directed to the 65kDa antigen of mycobacteria reacted with an additional band with a molecular weight of 58kDa in the control *E. coli* extracts. This behaviour is shown by three monoclonal antibodies which map at different regions along the protein (Fig. 2) and partial purification of the cross-reactive antigen from *E. coli* suggests that the same protein is recognized in each case. Rabbit antisera raised against synthetic peptides from different regions of the 65kDa sequence also reacted in western blots, again with an *E. coli* protein of 58kDa (Fig. 2). This analysis suggests that regions of cross-reactivity between the 65kDa antigen and an *E. coli* protein occur along most of the length of the molecule. Other monoclonal antibodies do not react with *E. coli*, indicating that these cross-reactive regions are interspersed with epitopes which show varying degrees of species specificity.

Cross-reactivity with *E. coli* also occurs at the level of T-cell recognition. Human T-cell lines generated to dif-

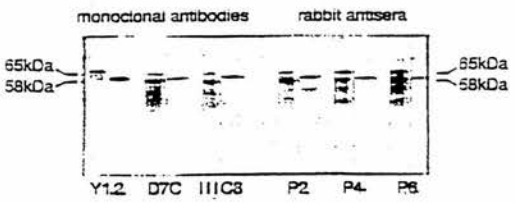

Fig. 2. Cross-reactivity of antibody epitopes.

Western blot analysis of M. tuberculosis (left hand lane) and E. coli (right hand lane). Monoclonal antibodies (ascitic fluid) were used at a dilution of 1 : 20 000 (Y1.2, IIIC3) or 1 : 100 000 (D7C). Sera from rabbits immunized with synthetic peptides P2, P4 or P6 were used at a dilution of 1 : 2500 for M. tuberculosis and 1 : 10 000 for E. coli. Sera from unimmunized rabbits showed no binding under these conditions.

ferent peptides from 65kDa sequence were tested for proliferation in response to extracts of *E. coli* fractionated by SDS-gel electrophoresis[16] (Fig. 3). A T-cell line which recognizes peptide 2 does not react with *E. coli*, while a line recognizing peptide 6 reacts with an *E. coli* component which has a molecular weight coincident with that of the protein identified by antibody cross-reactivity. While some of the T-cell determinants of the 65kDa antigen are cross-reactive with *E. coli*, others are more restricted in their distribution and a T-cell clone which discriminates between the 65kDa antigens of *M. leprae* and *M. tuberculosis* has been reported[15].

Homology with the *E. coli ams* gene

Mutation in the *ams* gene results in an extended half-life for messenger RNA in *E. coli* and a gene encoding a protein which complements this mutation has been cloned and sequenced[17]. Residues 296–420 of the mycobacterial 65kDa protein show a 65% homology with the sequence derived from the *ams* gene (Fig. 4). This sequence homology includes the region of the 65kDa antigen recognized by monoclonal antibody D7C as well as peptide 6 which has an associated T and B cell cross-reactivity. The 17kDa molecular weight of the *ams* gene product[17] is not, however, consistent with the western blot data and does not account for the more extensive cross-reactivity associated with other parts of the molecule. It is possible that the clone which has been sequenced may code for only a portion of the complete ams protein. Translation of the nucleotide sequence before the proposed AUG start signal in the *ams* gene[17] would result in a protein sequence in which six out of nine amino acids were identical to the corresponding region of the 65kDa antigen.

Cross-reactivity with other bacteria

The extent of the observed sequence homology between organisms as distantly related as mycobacteria and *E. coli* suggests that at least portions of the 65kDa antigen are under strong selective pressure for conservation and it is therefore anticipated that similar structures will be found in a wide range of bacteria. Quantitative analysis of cross-reactive binding of anti-65kDa monoclonal antibodies to soluble extracts from several non-mycobacterial genera showed marked differences between the tested species (Table 1). It is interesting that the variation in antigenic homology was expressed for the individual epitopes in most parts of the protein sequence.

Significance of the 65kDa antigen

While T lymphocytes capable of recognizing the 65kDa antigen are readily demonstrable, it is not known whether these cells play any role (stimulatory or suppressive) in protective immunity to mycobacterial infection or in the immunopathology of the disease. Analysis of the effect of the 65kDa antigen in animal models using the cloned gene in vaccinia virus and attenuated *Salmonella* vectors will provide important data in this respect.

From the standpoint of immune recognition, the 65kDa antigen of mycobacteria has become one of the most thoroughly characterized bacterial proteins. Its antigenic determinants are as well studied as those of the intensively studied bacterial toxins and adhesion factors[18]. The fact that many individuals appear to have primed T cells which recognize peptide sequences from the 65kDa antigen makes it an attractive model for carrying out basic research into the human immune response to intracellular bacteria in a manner comparable with that in which, for example, haemagglutinin and ribonucleoprotein from the influenza virus have been used as model studies for viral antigens (reviewed in Ref. 19). Some of the questions which studies of the 65kDa antigen may be able to answer include: is recognition of particular epitopes uniquely associated with specific HLA types? Do epitopes recognized by antibodies, helper T cells and cytotoxic T cells overlap, or are particular epitopes associated with single immune functions? Can a single epitope induce helper or suppressive effects in different individuals?

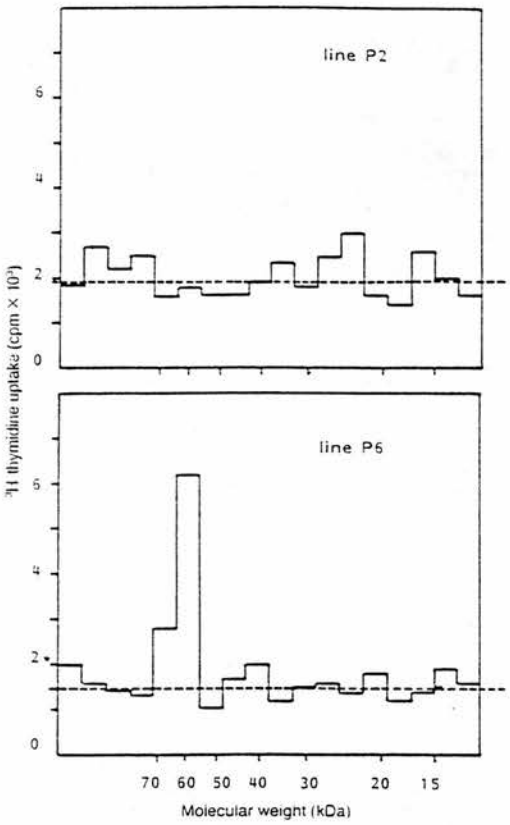

Fig. 3. *Cross-reactivity of T-cell epitopes.*

Proliferative response of T-cell lines reactive with synthetic peptides P2 and P6 to different molecular weight components of E. coli.

Nitrocellulose strips coated with different molecular weight fractions from an E. coli *extract were prepared by electroblotting[16] and added to proliferation assays after conversion to microparticulate form[22]. T-cell lines reactive with peptides P2 and P6 were generated from the ascitic effusion of a tuberculosis patient[23]. Proliferation as correlated with uptake of tritiated thymidine is shown as the mean of triplicate wells for each fraction. The broken line shows thymidine uptake in control wells without antigen. Thymidine uptake in response to the homologous peptide and to an extract of* M. tuberculosis *was 7884 ± 23 (c.p.m. ± SEM) and 11 258 ± 15 for line P2, and 16 698 ± 3 and 14 494 ± 19 for line P6.*

rostrum

Table 1. Cross-reactivity of 65kDa epitopes with diverse bacterial genera

Bacterial species	Monoclonal antibody					
	C1.1	IIH9	ML30	D7C	IC3	IIIC3
Nocardia asteroides	++++	++++	++	++++	++++	++++
Propionibacterium jensenii	++++	++	+	++++	+	++
Neissena flavescens	++++	++++	+	++	++++	++
Coryneobacterium pseudotuberculosis	+	+	++	++	+	+
Pseudomonas aeruginosa	++++	+++	+	++	+	+
Acinetobacter calcoaceticus	+	+	+	++++	+	+
Haemophilus influenzae B	+++	+	+	+	+	+
Streptococcus pneumoniae	++	++	+	+	+	+
Staphylococcus caseolyticus	++	+	+	+	+	+
Streptomyces albus	+++	+	+	+	+	+
Actinomyces viscosus	+++	++	+	+	+	+

Binding of monoclonal antibody (– : <10%, + : 10–25%, ++ : 25–50%, +++ : 50–75%; ++++ : >75%) to microtitre plates coated with soluble extracts from bacilli, relative to binding with *M. tuberculosis* (100%).

In view of the depth of knowledge about the 65kDa protein as an antigen, it is exciting to have an indication of the possible function of this protein within the bacterial cells. The marked sequence homology with the *E. coli ams* gene product strongly suggests that the 65kDa protein may be involved in the processing of a messenger RNA and an association with ribosomes and translation mechanisms seems possible.

Why does the 65kDa antigen elicit such strong immune responses? Is this a direct consequence of its presence in a diverse range of bacteria with constant restimulation of helper T cells during contact with normal bacterial flora? Or is there some particular feature of this protein which renders it strongly antigenic? If the immune response is due to priming by a cross-reactive bacterial antigen it would be appropriate to consider what effect exposure to other bacteria in endemic areas might have on the immune response to mycobacterial infection and how such factors might influence the effectiveness of BCG vaccination[20]. It may also be questioned whether priming of responses to the 65kDa antigen during mycobacterial infection or BCG vaccination has an effect on the subsequent immune response to challenge with other environmental bacteria. Is it possible that an individual who has been highly primed to the mycobacterial 65kDa antigen may mount an exaggerated and potentially harmful response to cross-reactive antigens from other essentially non-pathogenic bacteria? Such a phenomenon could provide an explanation for the occurrence of autoimmune diseases with no obvious aetiology, and would be consistent with the reported association of T-cell responses to mycobacterial antigens with rheumatoid arthritis[21].

A further application of the identification of a defined bacterial antigen with the ability to stimulate proliferation of helper T cells would be to use portions of this protein as a 'carrier' for inducing immune responses to other antigenic determinants. For example, a peptide of the 65kDa antigen which stimulates helper T cells could be coupled to peptides from other proteins containing the epitopes recognized by particular antibodies or cytotoxic T cells. Such a strategy may provide a basis for developing efficient 'subunit vaccines' capable of priming or eliciting the appropriate arms of the immune response.

The existing detailed information about the 65kDa antigen provides an opportunity to ask fundamental questions about the human immune response to intracellular bacteria which have applications well beyond the immediate field of mycobacterial disease.

Fig. 4. *Homology with the E. coli ams gene.*

The single letter amino acid code for a portion of the M. leprae 65kDa gene[6] is shown (upper line) along with the sequence derived for the E. coli ams gene[17] (lower line). Residues in the M. tuberculosis 65kDa protein[2] which differ from the M. leprae sequence are shown above the upper line. Residues denoted by small letters are outwith the proposed translated region of the ams gene[17]. Sequence homology is shown by the boxed residues.

We are grateful to R.A. Harrison and A. Vyakarnan for carrying out the sequence homology search; to R.A. Young for providing λgt11 sublibrary clones; to J.B. Rothbard for preparation of synthetic peptides; to T.M. Buchanan and B.R. Bloom for providing monoclonal antibodies; and to F. Salisbury for providing bacterial species.

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Antibody-mediated graft versus host reactions in renal transplantation

William M. Baldwin, III and Fred Sanfilippo

Graft versus host reactions (GvHR) are frequent and severe complications of bone marrow transplantation. Although the major symptoms of GvHR in these patients are thought to be mediated through T lymphocytes, both clinical and experimental observations indicate that antibody production also is stimulated by GvHR. In animal models of GvHR, Gleichmann and colleagues[1] have demonstrated a range of antibody-mediated lesions, which become more pronounced when the antigenic differences between donor and host favor stimulation of helper T lymphocytes and a chronic disease process.

Only limited attention has been given to the possibility that 'passenger lymphocytes' in solid tissue transplants may cause GvHR. In 1953 Simonsen suggested that the plasma cells he observed in the interstitium of transplanted canine kidneys were possibly of donor origin, but he abandoned this hypothesis when radiolabelling experiments demonstrated that cells of host origin infiltrated skin and kidney allografts (reviewed in Ref. 2). Yet, sporadic cases have been reported of unexpected hemolytic episodes following the transplantation of kidney, liver or lung from a donor who is Rh negative or O blood group to a patient whose erythrocytes bear Rh, A, B, or AB antigens[3–10]. One report suggested that irradiation of the donor kidney prevented antibody production to the recipient's erythrocytes[5], and in a few cases antibodies that were eluted from the recipients' erythrocytes have been shown to be of donor allotype[9–11]; thus substantiating the hypothesis that the hemolytic episodes reflect GvHR. Some investigators[5,6,11] have argued that this phenomenon may be more frequently encountered when cyclosporine A (CyA) is used as an immunosuppressive agent, because selective effects of CyA on T lymphocytes might prevent rejection of the donor B-lymphocytes but not suppress antibody production by these lymphocytes. Several recent reports have documented the longitudinal titers of circulating antibodies to recipient A, B or Rh antigens in CyA treated renal transplant recipients. These antibodies have been detected within 1 to 3 weeks after transplantation and they can persist for months[6], even after immunosuppression is discontinued[8]. Both IgG and IgM antibodies have been detected in the serum and in eluates from the recipients' erythrocytes by the indirect antiglobulin test which has a detection limit of about 10–200 ng/ml (Ref. 12). Assuming that a B lymphocyte can secrete about 2 × 10^8 IgG molecules (or 0.05 ng immunoglobulin) per day[13], then about 10^6–10^7 B lymphocytes with the appropriate specificity would be required to produce detectable levels of hemagglutinins in the circulation. Hemolytic anemia as a result of antibody production appears to be more frequent following transplants of liver than kidney[7,14]; this may simply reflect a dose effect of passenger B lymphocytes related to the size of the organ that is transplanted.

While antibodies that cause hemolysis have obvious practical consequences for some patients who receive an ABO or Rh incompatible transplant, the basic observation that B-lymphocytes which are passively transferred in a transplant can expand and produce clinically significant amounts of antibodies has broader implications. Antibodies to widely distributed antigens of a recipient such as those to HLA-A or -B antigens, might be absorbed from the circulation without causing specific clinical effects. Alternatively they could form soluble immune complexes that might deposit in the renal transplant and ultimately contribute to the peculiar form of allograft reaction described as transplant glomerulopathy[15,16]. Antibodies to antigens with a more restricted distribution might impair the function or reduce the number of certain cell populations. For example, antibodies directed against recipient class II MHC antigens might decrease the number of activated T lymphocytes or certain host antigen presenting cells. Even more specific antibodies such as anti-idiotypic antibodies could inhibit responses of certain critical clones of lymphocytes in the recipient. Indeed some of these antibody responses by donor lymphocytes may be responsible for the variable results observed in association with blood transfusions to kidney donors[17–19].

Antibodies of donor origin which are not directed to the recipient's own antigens might also be of importance. Misleading serological data could be caused by antibodies produced by donor lymphocytes to viral antigens. Based on increases in titers to cytomegalovirus (CMV), for example, it is generally believed that renal transplants from donors who are serologically positive for CMV almost always transmit CMV infections to the recipient[20], but some of these recipients have no symp-

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International Immunology, Vol 1, No. 2

Stress proteins may provide a link between the immune response to infection and autoimmunity

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Key words: heat-shock proteins, mycobacterial antigens, T cells, epitope mapping

Abstract

Stress proteins are frequently the target of humoral and cell-mediated immune responses to infection. These proteins belong to highly conserved gene families and there is substantial sequence homology between antigens produced by pathogenic organisms and the corresponding proteins from mammalian cells. Human T cells from sites of infectious and autoimmune lesions proliferate in response to stress proteins, and mapping of antigenic determinants on a mycobacterial stress protein shows that both species specific and highly conserved, 'self-like', regions of the molecule can take part in immune recognition. It is proposed that the lymphocyte population induced in response to stress proteins of pathogens during infection includes cells capable of autoimmune recognition of the corresponding self protein. Local accumulation of self stress proteins—in response to viral infection, for example—may subsequently provide a stimulus for proliferation of such autoreactive lymphocytes, thereby triggering a cycle of events which may contribute to the pathological damage associated with autoimmune disease.

Introduction

Exposure of cells to stress stimuli, such as an increase in temperature or exposure to toxic chemicals, results in induction of the synthesis of stress, or 'heat-shock', proteins (1). Stress proteins and structurally related proteins are also synthesized constitutively and perform essential functions during normal cell growth. Members of the 70 kilodalton heat-shock protein family (hsp70) are involved in membrane translocation (2,3), and the 90 kilodalton heat-shock protein (hsp90) has been found to associate with steroid hormone receptors (4,5). The product of the *groEL* gene of *Escherichia coli* is a major stress protein which belongs to a class of proteins, termed molecular chaperones, which assist in the assembly of multi-subunit complexes (6). Stress proteins have been highly conserved during evolution and members of each of the three protein families—hsp70, hsp90 and GroEL—are found in all cells, from bacteria to man (1,6,7 – 10).

Analysis of cell-mediated and humoral responses to a variety of bacterial and parasitic pathogens has shown that members of stress protein families are often strongly immunogenic during infection (11 – 18). Proteins involved in antibody responses to parasitic diseases (malaria, schistosomiasis, leishmaniasis, trypanosomiasis and filariasis) have been identified as members of the hsp70 and hsp90 gene families, and antigens belonging to hsp70 and GroEL families have been shown to play a role in T cell and B cell recognition during bacterial infections including leprosy, tuberculosis and Q fever. Immune responses to stress proteins have also been reported during autoimmune diseases. The mycobacterial GroEL stress protein has been identified as the target of a T cell clone capable of causing autoimmune disease in a rat model of adjuvant-induced arthritis (19), and elevated responses to the same protein have been found by testing T cells from the synovial infiltrates of rheumatoid arthritis

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Transmitting editor: Barry R. Bloom

Received 17 January 1989, *accepted* 31 January 1989

patients (20). Autoantibodies to hsp90 have been reported in systemic lupus erythematosus (SLE) (21) and elevated antibody responses to hsp70 and GroEL stress proteins have been found in SLE and in rheumatoid arthritis (22).

These reports indicate that stress proteins provide particularly attractive targets for immune recognition. Since stress proteins from different pathogens possess a high degree of sequence homology, it can be proposed that their apparent immunodominance is a reflection of repeated challenge to the immune system resulting in a high frequency of lymphocytes directed to conserved antigenic determinants. On the other hand, since these antigens share extensive sequence identity with the corresponding host cell proteins, their immune recognition must be influenced by mechanisms of 'self – non-self' discrimination, and it would be anticipated that response to conserved determinants will be excluded by tolerance. In order to evaluate the significance of immune responses to stress proteins during infection and autoimmune disease, it is important to establish whether or not conserved elements in these proteins can take part in immune recognition. In this paper we describe analysis of the cross-reactivity of T cell responses to stress proteins, and demonstrate the presence of human T cells capable of autoimmune recognition of conserved determinants. A model is proposed in which immune responses to stress proteins provide a link between infectious and autoimmune diseases.

Materials and methods

Preparation of antigens

Mycobacterial GroEL—the 65 kilodalton antigen (11)—was isolated from a recombinant *E.coli* strain (23) and was a gift from Dr Jan van Embden (RIVM, Bilthoven, The Netherlands). *E.coli* GroEL was purified from a recombinanant strain transformed with plasmid pND5 (11) using an adaptation of the method described by Chandrasekhar *et al.* (24). Purity was assessed by non-denaturing and SDS-polyacrylamide gel electrophoresis. Human GroEL was partially purified from heat-shocked peripheral blood mononuclear cells (PBMC) using DEAE-Sephacel and Sephacryl S200 chromatography. It was identified by its molecular weight under non-denaturing conditions (~ 850 kilodaltons), its subunit molecular weight (~ 60 kilodaltons), and by its cross-reactivity with antisera to *E.coli* GroEL and *Tetrahymena* hsp58 (9).

The hsp70 proteins were obtained from (*a*) a recombinant *E.coli* strain which overexpressed the mycobacterial 71 kilodalton antigen (25), (*b*) *E.coli* strain TG1, and (*c*) heat-shocked human PBMC. The proteins were purified using a modification of the ATP-agarose affinity chromatography procedure of Welch and Feramisco (26) and purity was assessed by SDS-polyacrylamide gel electrophoresis and Western blotting as described previously (25).

Synthetic peptides corresponding to T cell epitopes on the mycobacterial GroEL protein were selected and prepared by solid phase synthesis as described previously (27).

Isolation and cloning of human T cells

Human T cell lines (T1, T2, T3 and T6) specific for peptides corresponding to sequences from the mycobacterial GroEL protein (p1, residues 65 – 85; p2, 116 – 137; p3, 153 – 171; p6, 390 – 412) were isolated from the ascitic effusion of a patient reactive with *M.tuberculosis* as described previously (27,28). Lymphoblasts, activated with peptide (10 micrograms/ml) for 7 days and enriched on a Ficoll – Paque gradient, were restimulated with peptide and autologous irradiated (5000 Rads) EBV-transformed B cells (1×10^5/ml), and maintained in culture as long-term lines. A T cell clone (T5) was isolated from PBMC (2.5×10^5) stimulated for 7 days with synthetic peptide p5 (corresponding to residues 195 – 219) (3 micrograms/ml) by limiting dilution in the presence of peptide, autologous irradiated (2500 Rads) PBMC and interleukin-2 (Lymphocult T, Biotest-Serum Institut GmbH, Frankfurt, FRG) (29). Before use in proliferation assays, the T cell clone and lines were rested for 6 – 8 days after the last addition of antigen-presenting cells (APC) and antigen.

In proliferation assays, T cells (5×10^4/ml) were stimulated with antigen in the presence of autologous irradiated PBMC (1.25×10^5/ml) or EBV-transformed B cells (5×10^4/ml) in complete medium (RPMI 1640 supplemented with 2 mM glutamine, 100 IU/ml penicillin and streptomycin) containing 5% screened A+ serum. After 3 days incubation, tritiated methyl thymidine ([^{3}H]-TdR) (1 microcurie/well: Amersham International, Amersham, Bucks) was added and the cultures harvested onto glass fibre filters 8 – 16 h later. Proliferation, as correlated with [^{3}H]-TdR incorporation, was measured by liquid scintillation spectroscopy.

The proliferative response of lymphocytes isolated from the synovial fluid of patients with arthritis was tested by resuspending cells at 5×10^4 cells per well in complete medium plus antigen and measuring uptake of [^{3}H]-TdR as described above except that cultures were incubated for 6 days prior to addition of thymidine.

Results

T cell recognition of defined epitopes on the mycobacterial GroEL antigen

The ascitic effusion of patients suffering from tuberculosis infection is a rich source of lymphocytes reactive with antigens from *M.tuberculosis* including the mycobacterial GroEL stress protein (27,28). Synthetic peptides corresponding to regions of the GroEL sequence carrying motifs related to known T cell epitopes (27) were used to stimulate proliferation of lymphocytes isolated from the ascitic fluid of a single individual and T cell lines (T1, T2, T3 and T6) directed to four separate epitopes were obtained (Table 1). Each of the lines was stimulated by the eliciting peptide and by the purified mycobacterial protein, but the cells showed no response to the heterologous peptides. In addition to the T cell lines shown in Table 1, lines were also raised from the same ascitic fluid to peptides p4 (residues 180 – 196) and p5 (195 – 219). Proliferation of the remaining two lines occurred in the presence of antigen-presenting cells alone and was independent of the addition of exogenous antigen. A T cell clone (T5) showing a specific proliferative response to the p5 peptide was derived from peripheral blood mononuclear cells of a blood donor with no known history of mycobacterial or autoimmune disease and is included in Table 1.

These results are in agreement with previous reports indicating that the mycobacterial GroEL antigen contains a broad array of epitopes available for recognition by human T lymphocytes

Table 1. Mapping of species specific and conserved T cell epitopes on the mycobacterial GroEL stress protein

	Antigen																									T cell response (SI)				
																										T1	T2	T3	T5	T6
p1	65																				85									
mycobacteria	Y	E	K	I	G	A	E	L	V	K	E	V	A	K	K	T	D	D	V	A	G					2.8	1.1	0.9	0.2	1.1
E.coli	F	*	N	M	*	*	Q	M	*	*	*	*	*	S	*	A	N	*	A	*	*									
human	*	K	N	*	*	*	K	*	*	Q	D	*	*	N	N	*	N	E	E	*	*									
p2	116																								137					
mycobacteria	K	R	G	I	E	K	A	V	D	K	V	T	E	T	L	L	K	D	A	K	E	V				1.0	8.1	1.7	0.5	1.1
E.coli	*	*	*	*	D	*	*	*	T	A	A	V	*	E	*	K	A	L	S	V	P	C								
human	R	*	*	V	M	L	*	*	*	A	*	I	A	E	*	K	*	Q	S	*	P	*								
p3	153																		171											
mycobacteria	D	Q	S	I	G	D	L	I	A	E	A	M	D	K	V	G	N	E	G							0.8	0.8	15.9	0.4	1.9
E.coli	*	E	T	V	*	K	*	*	*	*	*	*	*	*	*	*	K	*	*											
human	*	K	E	*	*	N	I	*	S	D	*	*	K	*	*	*	R	K	*											
p5	195																								219					
mycobacteria	K	G	Y	I	S	G	Y	F	V	T	D	A	E	R	Q	E	A	V	L	E	E	P	Y	I	L	1.0	1.4	0.9	12.9	1.1
E.coli	R	*	*	L	*	P	*	*	I	N	K	P	*	T	G	A	V	E	*	*	S	*	F	*	*					
human	R	*	*	*	*	P	*	*	I	N	T	S	K	G	*	K	C	E	F	Q	D	A	*	V	*					
p6	390																						412							
mycobacteria	R	K	H	R	I	E	D	A	V	R	N	A	K	A	A	V	E	E	G	I	V	A	G			0.8	1.4	0.9	1.3	33.1
E.coli	K	*	A	*	V	*	*	*	L	H	A	T	R	*	*	*	*	*	*	V	*	*	*							
human	K	*	D	*	V	T	*	*	L	N	A	T	R	*	*	*	*	*	*	*	*	L	*							
	mycobacterial GroEL																									4.0	3.1	6.7	9.4	8.1
	E.coli GroEL																									1.0	0.7	4.2	9.2	4.4
	human GroEL																									1.1	1.0	0.7	9.6	8.9

Five epitopes involved in human T cell recognition of the mycobacterial GroEL protein were mapped using synthetic peptides. Two T cell lines (T1 and T2) were specific for the mycobacterial antigen, while T cell line T3 was also able to recognize the related protein from *E.coli*. In addition to the bacterial GroEL antigens, T cell line T6, and T cell clone T5, were stimulated by the related protein purified from human cells.

The sequence of each epitope is shown using the single letter code for amino acids along with the corresponding regions of *E.coli* GroEL (6) and a member of the GroEL family from human mitochondria (10). Residues identical to the mycobacterial protein are shown as an asterisk.

Maximum proliferation in a dose response curve (0.3 – 30 micrograms/ml) is shown. Results are expressed as stimulation index (SI).

SI = (T cells + APC + antigen)-(T cells + APC)/(T cells + APC).

(27,30). An unexpected finding is that T cells capable of recognition of many different determinants (at least four out of the six peptides tested) could be isolated from a single individual.

Cross-reactivity of T cell responses

Having established a panel of lymphocyte populations responsive to individual defined epitopes on the mycobacterial GroEL antigen, it was of interest to determine whether or not the homologous stress proteins purified from other organisms could stimulate these cells. The proliferative response of each T cell preparation was therefore tested using GroEL purified from *E.coli* and a partially purified preparation of a GroEL protein from human cells.

The corresponding GroEL protein from *E.coli* was recognized by T cells directed to epitopes defined by synthetic peptides p3, p5 and p6, with the latter two determinants also being conserved in GroEL extracted from human cells. A human mitochondrial protein (P1) has recently been sequenced, and comparison with the mycobacterial GroEL sequence indicates that P1 belongs to the GroEL family (10). Comparison of amino acid sequences in the regions of each of the three GroEL proteins corresponding to T cell epitopes (Table 1) shows that immunological cross-reactivity does not require complete sequence identity, but presumably reflects conservation of key residues involved in interactions with components of the major histocompatibility complex (MHC) and with the T cell receptor. A synthetic peptide corresponding to the appropriate sequence of the related human protein stimulated proliferation of the T cell clone directed to the p5 epitope (residues 195 – 208) of mycobacterial GroEL (Fig. 1) thus confirming the autoreactivity associated with this area of the protein.

Recognition of stress proteins by T cells during autoimmune disease

An association between rheumatoid arthritis and lymphocyte responses to mycobacterial extracts has been reported (31) and we therefore tested for recognition of stress proteins by T cells from arthritis patients. Lymphocytes obtained from synovial infiltrates showed strong responses to stess proteins (Table 2); peripheral blood lymphocytes from the same patients gave only weak proliferation (data not shown). The response could be inhibited by addition of anti-CD3 antibodies, showing that it was an indication of T cell proliferation. Stress proteins (hsp70 and GroEL) from mycobacteria were most effective in stimulating T cell proliferation, but the same cells also responded to the homologous proteins from *E.coli*. Lower, but clearly detectable

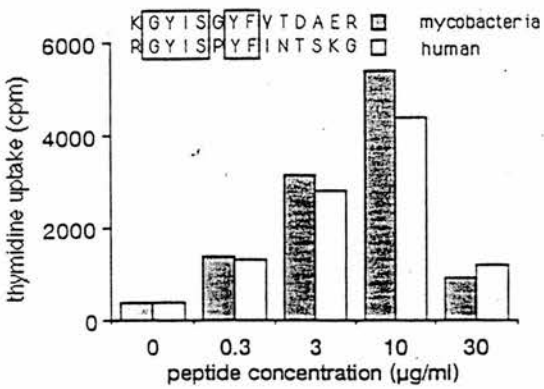

Fig. 1. A human T cell clone responds to a synthetic peptide corresponding to a sequence from the human GroEL protein. The epitope recognized by T cell clone T5 was localized within the region defined by residues 195 – 208 of the mycobacterial GroEL. A synthetic peptide corresponding to the equivalent region of the human GroEL sequence was prepared and shown to stimulate a proliferative response comparable to that induced by the mycobacterial peptide. Only 6 of the 14 residues are identical between the two peptides.

Table 2. Lymphocytes isolated from the synovial effusion of arthritis patients proliferate in response to stress proteins

Antigen	Source	T cell response (thymidine uptake) patient number		
		1	2	3
hsp70	mycobacteria	5095	19 244	2679
	E.coli	4205	13 006	2048
	human	2209	5891	1143
GroEL	mycobacteria	8077	22 076	2189
	E.coli	1095	7447	1285
medium control		847	562	522

The proliferative response to stress proteins was tested using lymphocytes isolated from the synovial effusion of two patients with rheumatoid arthritis (patients 1 and 3), and one patient with reactive arthritis (patient 2). Patients 2 and 3 were typed as HLA DR2,5 and HLA DR2,3 respectively. The tissue type of patient 1 has not been determined. All of the patients showed a strong response to the mycobacterial stress proteins and, particularly in the case of patient 2, marked response to the corresponding human hsp70 protein was also observed.

The results are expressed as mean counts per minute (cpm) for triplicate cultures. The standard errors were less than 20%. The maximum response in a dose response curve was obtained at 10 micrograms protein/ml and is shown.

responses were observed when hsp70 from human cells was used as an antigen. No response to human hsp70 was found when testing peripheral blood lymphocytes from normal donors. Preliminary experiments using lymphocytes from osteoarthritis patients showed no elevated responses to stress proteins (B. R. Bloom, personal communication). These results demonstrate that stress proteins are recognized by patients with autoimmune disease and that a portion of the T cell population involved in the human immune response to stress proteins is capable of recognizing the homologous 'self' proteins.

Discussion

In assessing the significance of immune responses to highly conserved antigens such as the stress proteins, it is important to determine whether the determinants involved in immune recognition are made up of conserved or variable amino acid residues. Species specific epitopes can be recognized as foreign antigens, but epitopes from conserved regions of the protein should be seen as 'self' and should not elicit an immune response. We report here that, in the case of the mycobacterial GroEL stress protein, the determinants available for human T cell recognition include both species specific and conserved elements and conclude that the mechanisms of self – non-self discrimination are not sufficiently stringent to exclude T cell recognition of 'self-like' epitopes of stress proteins.

The relative contribution of conserved and variable determinants to the overall immune response has not yet been assessed and it is possible that, as has been reported for antibody responses to stress proteins (32), the normal cellular response to such antigens is dominated by recognition of species specific epitopes. An autoreactive component is, however, detectable in the proliferative response of bulk lymphocytes isolated from the synovial fluid of arthritis patients. Antigen recognition by T cells requires interaction with an MHC molecule on the surface of an antigen-presenting cell, and specificity associated with the antigen-binding site on the MHC molecule will result in preferential recognition of different epitopes on stress proteins by individuals with different MHC haplotypes. The relative frequency of presentation of variable and conserved epitopes will therefore be regulated in part by MHC polymorphism.

The presence of T cells capable of responding to a self protein need not necessarily result in autoimmune pathology. Indeed, the T cell clone (T5) recognizing the self peptide from the GroEL protein was isolated from an individual with no clinical history of autoimmune disease. Proliferation of autoreactive T cells may be restricted *in vivo* by the action of regulatory pathways perhaps involving suppressor or cytotoxic cells. Alternatively, the self protein may not be efficiently processed and presented at sufficiently high antigen concentration for lymphocyte recognition *in vivo*. If the latter mechanism plays a role in tolerance to self proteins, then stimuli which cause increased synthesis of stress proteins may lead to antigen concentrations sufficient to stimulate autoreactive lymphocytes. A variety of physiological events, such as viral infection (33 – 36) or possibly stresses occurring within inflamed joints (37), could increase the local concentrations of stress proteins in this way. Alternatively, localized changes in the level of expression of MHC molecules could result in increased presentation of self stress proteins (38).

Based on three distinctive features of stress proteins—immunogenicity, sequence conservation and differential expression—we propose that there may be a link between their role in infection and a possible involvement in autoimmunity. Immune responses to stress proteins are generated during bacterial and parasitic infections. This response will be directed primarily to variable epitopes but, under some circumstances (possibly related to the nature of the infection and to the MHC

haplotype) an immune response to self-like determinants may also be induced. A subsequent local increase in synthesis of the corresponding self stress protein, for example in response to viral infection, could activate the autoreactive lymphocyte population and provide a focus for generation of autoimmune pathology. This model may provide an explanation for the reported links between autoimmune disease and a diversity of bacterial and viral infections (39), and could also be relevant in understanding the immunopathology which is often associated with chronic bacterial and parasitic diseases.

This report demonstrates that a human T cell response to self stress proteins does occur. In order to validate the model proposed above it will be necessary to show that alterations of stress protein synthesis *in vivo* can result in activation of this potentially autoreactive T cell population. Animal models of autoimmune disease (19) provide a possible approach to further evaluation of this model.

Acknowledgements

We are grateful to Dr Jan van Embden for supply of purified mycobacterial GroEL, to Dr Richard Hallberg for providing antibody to *Tetrahymena* hsp58 and to Dr Sara Brett for helpful discussion. R. A. Y. is a Burroughs Wellcome Scholar in Molecular Parasitology. P. M.-S. is a Wellcome Overseas Fellow.

Abbreviations

APC	antigen-presenting cells
ATP	adenosine triphosphate
cpm	counts per minute
EBV	Epstein Barr virus
hsp70	70 kilodalton heat-shock protein family
hsp90	90 kilodalton heat-shock protein family
MHC	major histocompatibility complex
PBMC	peripheral blood mononuclear cells
SDS	sodium dodecyl sulphate
SLE	systemic lupus erythematosus
TdR	thymidine

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1.2 ANALYSIS OF THE ANTIGEN SPECIFICITY OF T CELL RESPONSES

1.2.3 HOUSE DUST MITE DERIVED ALLERGENS

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Cloned human T lymphocytes reactive with *Dermatophagoides farinae* (house dust mite): a comparison of T- and B-cell antigen recognition

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Accepted for publication 17 July 1987

SUMMARY

In this report, T-cell and B-cell recognition of the house dust mite *Dermatophagoides farinae* (*D. far.*) is compared. Nitrocellulose immunoblots of polyacrylamide gel electrophoresis (SDS–PAGE)-fractionated *D. far.* were added to proliferation assays to map the antigen specificity of cloned human helper T cells and a long-term line induced with *D. far.* T-cell recognition was of a polypeptide of molecular weight 9000–13.000, that migrates with the serologically defined allergen *Der f* II (12.500 MW). Since the cloned T cells, unlike the polyclonal response, failed to respond to *Dermatophagoides pteronyssinus* (*D. pter.*), this suggests that they recognize a species-specific epitope. In contrast, analysis of the B-cell response using Western blotting demonstrated that, in addition to *Der f* II, antibodies reactive with the major allergens *Der f* I (26.000 MW) and *Der f* III (29.000 MW) were present in the serum. Similar specificities were seen in the antibody response to *D. pter.*, and while it has been reported that the B-cell response to *D. far.* and *D. pter.* are predominantly cross-reactive, our observations suggest that species-specific CD4-positive T cells are present in the overall cellular response to *D. far.*

INTRODUCTION

The T-cell dependency and reaginic activity of IgE in clinical allergy is well documented and, unlike other classes of immunoglobulin, rather than being protective is responsible for allergic sensitivity in atopic individuals (Ishizaka, 1984). Early studies (Orange, Austen & Austen, 1971) demonstated that allergen–IgE antibody reactions in the human lung induce chemical mediators that cause the symptoms of respiratory allergy.

In contrast, the antigen specificity and functional role of T lymphocytes in the induction and regulation of allergic respiratory diseases remain ill defined, with current research restricted largely to phenotypic analysis (Metzger *et al.*, 1987). Recognition of allergens such as ragweed (Meuer *et al.*, 1983), pollen (Brostoff, Greaves & Roitt, 1969) and *Dermatophagoides* (Rawle, Mitchell & Platts-Mills, 1984; Lanzavecchia *et al.*, 1983) by the T cells of atopic individuals has been reported. Rawle *et al.* (1984) observed that helper T cells from atopic but not control individuals responded to the purified major allergen pI from the house dust mite *D. pter.* Lanzavecchia *et al.* (1983) isolated HLA-DR-restricted T-cell lines and clones reactive with the aeroallergen *D. pter.* They subsequently used alloreactive T-helper clones for the *in vitro* stimulation of IgE production (Lanzavecchia & Parodi, 1984).

Investigation of the antigenic structures of the house dust mites *D. pter.* and *D. far.* suggest that *Der p* I and *Der f* I, both of molecular weight 24.000, are the major allergens (Chapman & Platts-Mills, 1980; Heymann, Chapman & Platts-Mills, 1986). Others have reported major allergens of slightly different molecular weights, but it has been accepted generally that the most allergenic fractions were distributed in the range of 20.000–30.000 (Dandeu *et al.*, 1982; Stewart & Turner, 1980). Additionally, *Der f* II, a major allergen (12.500 MW), and other minor allergens have also been described (Heymann *et al.*, 1987). However, the identification of these antigens was based on monoclonal antibodies, and since T and B cells may recognize different components of *D. far.*, this should be supplemented by a direct approach for the analysis of T-cell antigen recognition.

In this study, in order to investigate the role of cell-mediated immunity in the allergic response to house dust mite, T-cell lines and clones reactive with *D. far.* were isolated. Using the SDS–PAGE immunoblot assay system for T-cell antigen recognition (Young & Lamb, 1986) the fine specificity could be mapped to determinants present in the molecular range of 9000–13.000 and

Abbreviations: APC, antigen-presenting cell; *D. far.*, *Dermatophagoides farinae*; *D. pter.*, *Dermatophagoides pteronyssinus*; [^{3}H]TdR, tritiated methyl thymidine; PBMC, peripheral blood mononuclear leucocytes; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis.

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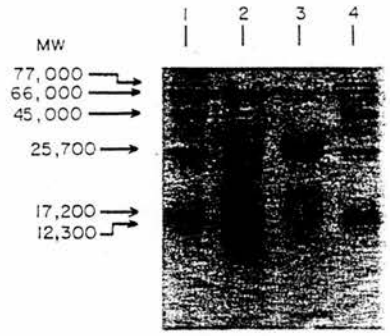

Figure 1. SDS–polyacrylamide gel of *D. far.* and *D. pter.* extracts. *D. far.* extract (10^5 BU protein per lane) was prepared and run on SDS–polyacrylamide gel, and proteins were blotted onto nitrocellulose. A representative gel stained with Coomassie blue is shown with molecular weight markers. *D. pter.* extract was run in parallel (*D. pter.*, Lane 3; *D. far.*, Lane 2; molecular weight markers, Lanes 1 and 4).

was specific for *D. far.*, showing no recognition of the closely related genus *D. pter.* In contrast, the IgE antibody response to *Der f* II was cross-reactive.

MATERIALS AND METHODS

Antigens

Lyophilized extracts of *D. far.*, Timothy grass, five-grass mix and Parietaria were kindly provided by Pharmacia (Uppsala, Sweden). *D. pter.* was the generous gift of Bencard (Brentford, Middlesex).

Antibodies

Monoclonal antibodies reactive with T-cell surface antigen, Leu 2a, Leu 3a, Leu 4, HLA-DR and IL-2 receptor were purchased from Becton-Dickinson, Sunnyvale, CA.

Preparation of immunoblots

SDS–PAGE immunoblots were prepared as described previously (Young & Lamb, 1986). Briefly, antigen was boiled in the presence of SDS and 2-mercaptoethanol and separated by SDS–polyacrylamide gel electrophoresis in a 'mini-gel' apparatus (Hoeffer Scientific, San Francisco, CA) using the Laemmli system (Laemmli, 1970) with an acrylamide concentration of 15% (w/v). Samples of *D. far.* extract for electrophoresis contained 10^5 biological units (BU) of protein per lane. Proteins were transferred from gels to nitrocellulose by electroblotting as described by Towbin, Staehelin & Gordon (1979). Blots were reversibly stained by dipping for a few seconds in Amido black (0·1% in 0·5% acetic acid) and washed with distilled water to permit accurate localization of protein lanes (Fig. 1). Blots were further washed with 0·1% Triton X-100 in phosphate-buffered saline (PBS) and then with PBS alone prior to use. Each nitrocellulose blot (0·5 × 4 cm) was divided into 20 equal sections. These sections were then solubilized using the modification of Abou-Zeid *et al.* (1987). Dimethylsulphoxide (1 ml) was added to each section for 1 hr at room temperature followed by an equal volume of bicarbonate/carbonate buffer, pH 9·6, to allow reprecipitation. The preparations were then precipitated in a microfuge at 10,000 r.p.m. and washed four times with RPMI-1640 medium (Flow, Irvine, Ayrshire) and resuspended to a final volume of 0·5 ml, of which 20 μl were added to each well in proliferation assays. Molecular weights were determined from a standard curve obtained using molecular weight markers (Sigma, Poole, Dorset).

Preparation of lymphocytes

Peripheral blood mononuclear leucocytes (PBMC) obtained from a healthy adult with perennial rhinitis and a positive skin prick test to the house dust mite *D. far.* were isolated by centrifugation on a discontinuous gradient of Ficoll–Paque (Pharmacia).

Isolation of antigen-reactive T-lymphocyte lines and clones

Antigen-reactive T-cell line and clones were isolated as described previously (Lamb *et al.*, 1982). Briefly, PBMC ($2·5 \times 10^5$/ml) were cultured with *D. far.* antigen (10^3 BU/ml) for 7 days in RPMI-1640 supplemented with 2 mM L-glutamine, 100 IU/ml penicillin/streptomycin and 5% screened AB^- serum. Lymphoblasts enriched on Ficoll–Paque were established as a long-term line in the presence of irradiated (2500 rads) autologous PBMC, *D. far.* and interleukin-2 (IL-2; Lymphocult T, Biotest Folex, Frankfurt, FRG) or cloned by limiting dilution from the line. For cloning, lymphoblasts (33⅓ cells/ml) were plated in Microtest II trays together with irradiated autologous PBMC (5×10^5/ml), *D. far.* (10^3 BU/ml) and IL-2 (Lymphocult T 5% v/v supplemented with recombinant IL-2, 5 U/ml, the generous gift of Boehringer, Mannheim, FRG). After 7 days, growing clones were transferred to flat-bottomed 96-well microtitre trays and subsequently to 24-well trays. At each transfer the clones received fresh IL-2 and irradiated autologous PBMC together with specific antigen (*D. far.*; 10^3 BU/ml). The clones were maintained with further IL-2 every 3–4 days, and irradiated autologous PBMC and *D. far.* were added every 7 days. Before use in proliferation assays, the clones were rested for 6–8 days after the last addition of filler cells and antigen.

Proliferation assays

T cells of the long-term line (DX2) and clone (DD11; 5×10^4/ml) were cultured with soluble or insoluble antigen in the presence of autologous irradiated PBMC (5×10^5/ml) in 96-well flat-bottomed microtitre plates, as described previously (Young & Lamb, 1986). After a 72 hr incubation, the cultures were pulsed with 0·66 μCi of tritiated methyl thymidine ([^{3}H]TdR; Radiochemicals Inc., Amersham, Bucks, U.K.) for 8–16 hr, then harvested onto glass-fibre filters.

Proliferation as correlated with [^{3}H]TdR incorporation was measured by liquid scintillation spectroscopy. The results are expressed as the mean counts per minute (c.p.m. ± SEM). The duration of unfractionated PBMC cultures (10^5/well) was 7 days.

Autoradiography from immunoblots of D. far. *and* D. pter.

Nitrocellulose-transferred SDS–PAGE immunoblots of *D. far.* and *D. pter.* were prepared as described. Western blotting and autoradiography were performed using the modification of Tee *et al.* (1987). Briefly, non-specific binding was blocked by immersing the nitrocellulose sheets in 2% human serum albumin in Tris buffer (pH 7·5) for 1 hr at 39°. After washing, a range of concentrations of autologous serum (undiluted, 1:2, 1:5) was allowed to react over the nitrocellulose sheets for 16 hr. After

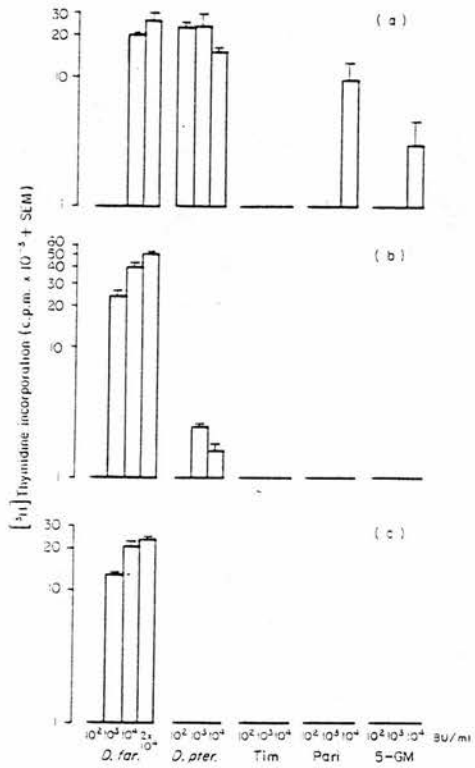

Figure 2. T-cell proliferative responses to a panel of soluble aeroallergens. (a) PBMC (10^5/well) were cultured with soluble *D. far.*, *D. pter.*, Timothy grass, Parietaria and five-grass mix. Proliferation was determined by [^{3}H]TdR incorporation in a 7-day assay. The results are expressed as counts per minute (c.p.m.) ± SEM of triplicate cultures. Background response of PBMC in the absence of antigen was 349 ± 48 c.p.m. (b) T cells of line (DX2) and (c) clone (DD11) (10^4 well) were stimulated with the same aeroallergens together with autologous irradiated PBMC (5×10^4/well). [^{3}H]TdR incorporation was determined at 72 hr. Background responses of the DX2 and DD11 to APC in the absence of antigen were 87 ± 19 and 95 ± 30 c.p.m., respectively.

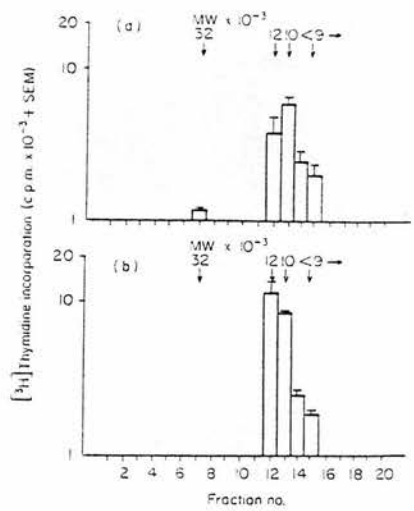

Figure 3. Differential pattern of reactivity of the T-cell line (a) and clone (b) to SDS–PAGE antigens solubilized from immunoblots of *D. far.* T cells of the *D. far.*-specific line (DX2) and the clone (DD11) (10^5/ml) were cultured with SDS–PAGE-separated immunoblots (20 fractions) after solubilization with DMSO, together with irradiated PBMC (5×10^5/ml) and assayed as described in the legend to Fig. 2b and c. Background response of DX2 and DD11 to APC in the absence of antigen was 973 ± 20 and 66 ± 16 c.p.m., respectively.

washing, this was followed by a further 16-hour incubation with the tracer ^{125}I anti-IgE (Pharmacia) (100 μl/approximately 10^4 c.p.m.). The washed, dried sheets were exposed to X-OMAT L Kodak film with Du Pont Quanta III intensifying screens at −70° for 48 hr, and then developed. Negative controls using serum (1:2 and 1:5) from a non-atopic individual were processed in parallel.

Cytofluorimetric analysis

T cells were incubated with monoclonal antibodies Leu 2a, Leu 3a, Leu 4, HLA-DR and anti-IL-2R followed by FITC-conjugated rabbit anti-mouse immunoglobulin ($F(ab)_2$; Dakopatts, High Wycombe, Bucks, U.K.). The cells were analysed for fluorescence on a FACS-analyser cytofluorimeter (Becton-Dickinson).

RESULTS

T-cell proliferation in response to soluble *D. far.* antigen

PBMC from a patient with perennial rhinitis proliferated in a dose-dependent manner when stimulated with soluble *D. far.* extract (Fig. 2a). In addition, proliferative responses were also observed to the other aeroallergens as compared to the medium control. Although no response was observed to Timothy, the biological activity of the allergen was confirmed in sensitized patients. Both the long-term T-cell line (DX2) and cloned T cells (DD11) proliferated in response to the inducing antigen *D. far.*, but failed to recognize the allergens Timothy, Parietaria and five-grass mix over a range of concentrations. DX2 gave a limited proliferative response to *D. pter.*, but DD11 showed no recognition of this allergen (Fig. 2b and c). This confirms the specificity of the T-cell line and clone for *D. far.*

Reactivity pattern of T-cell line and clone to SDS–PAGE immunoblots of *D. far.*

T lymphocytes from the line and clone in the presence of accessory cells were observed to proliferate in response to *D. far.* supplied in particulate form bound to nitrocellulose after fractionation on SDS–PAGE (Fig. 3). The long-term T-cell line (DX2) predominantly recognized antigenic determinants within the molecular weight range 9000–13,000 (Fig. 3a) which contained the only determinant recognized by the clone DD11 (Fig. 3b). In addition, the line showed minimal recognition of the protein band (24,000–29,000) that would correspond to the

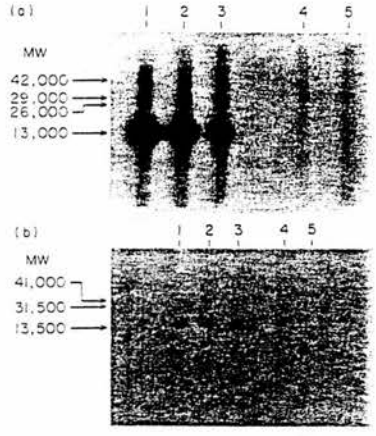

Figure 4. Autoradiographs of Western blots showing the IgE response of autologous and non-atopic allogeneic sera to *D. far.* and *D. pter.* antigens. *D. far.* (a) and *D. pter.* (b) mite body extracts were separated by SDS–PAGE and transferred to nitrocellulose. The strips were probed with sera from the mite-allergic PBMC donor (undiluted. Lane 1; 1:2. Lane 2; and 1:5. Lane 3) and from a non-allergic control (1:2. Lane 4; and 1:5. Lane 5) and autoradiographed to detect binding of IgE as described in the Materials and Methods.

Der f I and *Der f* III allergens. To control for the possibility that proliferation was a non-specific mitogenic effect, all 20 fractions were added in the presence of accessory cells to cloned T cells reactive with influenza haemagglutinin (Lamb & Green, 1983), with the result that no proliferation was observed. Neither were any of the fractions able to inhibit the response of this clone to influenza, thus eliminating the presence of non-specific inhibitors (data not shown). These results would suggest that the proteins in the molecular weight range 9000–13,000 constitute the major component of T-cell recognition for this individual.

Reactivity pattern of specific IgE to immunoblots of *D. far.*

The autoradiographs using autologous serum showed strong IgE binding at the 13,000 fraction corresponding to the major site of T-cell recognition (Fig. 4a). Fainter bands were demonstrable at 26,000, 29,000 and 42,000 MW. These first two bands correspond to the molecular weights of *Der f* I and *Der f* III, and reflect the minor recognition site of the T-cell line DX2. Similarly, the pattern of binding to *D. pter.* was predominantly against the 13,500 MW band, with additional bands at 41,000 and 31,500 (Fig. 4b).

Interestingly, the major protein band on this gel was consistent with *Der p* I (Fig. 1). None of these antigens was recognized by the control sera.

Phenotypic analysis of cloned T lymphocytes

Since the T cells showed identical specificity to the anti-*D. far.* IgE response (Fig. 4a), it was of interest to determine if those T cells were of the helper phenotype. Both the long-term T-cell line DX2 and clone DD11 expressed CD3 and MHC class II determinants characteristic of activated mature peripheral T cells. The clone DD11 was CD4-positive and therefore of the helper/inducer population. The line was also predominantly CD4-positive, although there was a minor component of CD8 (suppressor/cytotoxic T subsets)-positive T cells. The reduced expression of IL-2R in clone DD11 as compared to the line may reflect the state of activation (IL-2R down-regulation) since the cells were rested for 6–8 days after the last addition of filler cells and antigen before phenotypic analysis (Fig. 5).

DISCUSSION

The T-cell dependency of antibody production in allergic disease is well established (Ishizaka, 1984). However, the specificity and regulatory role of T cells in the immune response to house dust mite remain to be explored.

In this study the antigen specificity of both the polyclonal and clonal response of a patient with perennial rhinitis attributable to house dust mite allergy has been analysed. At the polyclonal level, both *D. far.* and the closely related house dust mite *D. pter.* were able to induce marked T-cell proliferation. Therefore, in order to determine whether or not T-cell recognition of *D. far.* was limited to cross-reactive determinants also present on *D. pter.*, an IL-2-dependent T-cell line and clone induced with *D. far.* were isolated. Similar to the polyclonal response, the T-cell line responded to both *D. far.* and *D. pter.*, although the magnitude of the response to the latter was considerably less, suggesting that T-cell recognition was directed mainly to species-specific determinant(s). Indeed, the cloned T cells also appeared to recognize a species-specific antigen of *D. far.*

The information available on T-cell recognition of the genus *Dermatophagoides* is limited. Rawle *et al.* (1984) described polyclonal T-cell proliferation to the pI antigen of *D. pter.*; however, whether or not the responses were cross-reactive with *D. far.* was not investigated. Certainly it would appear that human IgE antibody response to *Der p* I and *Der f* I is predominantly cross-reactive (80–95%: Chapman, Heymann & Platts-Mills, 1987), and although T and B cells may recognize different determinants within a protein antigen (Berzofsky, Richman & Killon, 1979: Lamb & Green, 1983), the specificity of the T-cell response may parallel that observed for B cells. In order to determine whether the T-cell response was directed towards *Der f* I, the reputed major allergen of *D. far.*, nitrocellulose immunoblots of SDS–PAGE-separated antigen were prepared and added to T-cell proliferation assays. Using this approach the determinant(s) recognized by both the T-cell line and clone could be mapped in the molecular range of 9000–13,000. This co-migrates with *Der f* II (MW 12,500), recently proposed by Heymann *et al.* (1987) to be a major allergen of *D. far.* in addition to the 24,000 MW protein *Der f* I (Heymann *et al.*, 1986). Interestingly, the results reported would suggest that the T-cell response of this individual is directed predominantly against a species-specific determinant(s) in *Der f* II. Whether or not *Der f* II, as opposed to *Der f* I for example, is an immunodominant T-cell allergen for the haplotype of this individual, such as has been described for the cytotoxic T-cell response to the internal components of influenza (Gotch *et al.*, 1987) warrants investigation. Alternatively, as suggested by the polyclonal response, T-cell recognition is neither limited to species-specific determinants, or indeed to *Der f* II, and

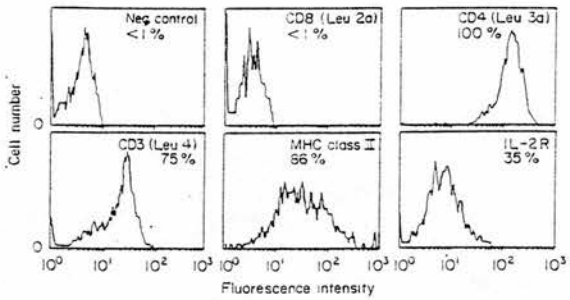

Figure 5. Indirect immunofluorescent cytofluorimetric analysis of clone DD11. Samples were analysed with monoclonal antibodies as indicated above the histograms. Percentage positivity is shown.

therefore the clonal response merely reflects the restriction element usage for a specific epitope.

In analysing the specificity of the *D. far.* IgE antibody response, similar to the T cells, recognition was predominantly of a protein that migrates with the same molecular weight as the *Der f* II allergen (12,500), although antibodies reactive with determinants that migrate with identical molecular weights to the f I and f III allergens (26,000 and 29,000) respectively (Heymann *et al.*, 1987) were also observed. These results are interesting for they suggest that while the T-cell recognition is mainly of the *Der f* II, allergen, the IgE antibody response is directed towards both the major and minor allergens. The human IgE antibody to *Der p* I and *Der f* I appears to be 80–95% cross-reactive (Chapman *et al.*, 1987), therefore it is possible that T cells primed to *D. pter.* are providing the helper activity for the cross-reactive B-cell response. Indeed, the PBMC of this individual were able to respond to *D. pter.* Whether or not the antibody response to *Der f* II and *Der f* III operates through the same mechanism is unclear. Alternatively, the *Der f* II-reactive T cells may provide not only specific helper activity for the anti-*f* II antibody response, but also 'intermolecular' help (Lake & Mitchison, 1977) for the anti-*f* I and *f* III B-cell response. These same arguments may apply to the immune response to *D. pter.* Furthermore, the fact that the cloned T cells and the line described here express membrane CD4 raises the possibility that they are able to regulate IgE synthesis, and therefore may be important in the pathogenesis of the allergic response to the house dust mite.

ACKNOWLEDGMENTS

Dr R. O'Hehir is the recipient of a Sir Robert Menzies Medical Scholarship. This work was supported by the Menzies Trust, Fisons Australia, the Asthma Foundation of Victoria, Australia and the Clinical Research Committee of the National Heart and Chest Hospital. We are grateful to the Department of Microbiology, Cardiothoracic Institute, for the generous use of facilities, to Miss A. Hartnell and Dr A. Frew for FACS analysis, to Dr R. Moqbel, Dr P. Fitzharris, Dr R. Knight, Mr D. Gordon and Mr P. Townsend for helpful scientific discussion, and to Katy Sowton for typing the manuscript.

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An *in vitro* model of allergen-dependent IgE synthesis by human B lymphocytes: comparison of the response of an atopic and a non-atopic individual to *Dermatophagoides* spp. (house dust mite)

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Accepted for publication 27 November 1988

SUMMARY

An allergen-dependent *in vitro* model of immunoglobulin E (IgE) synthesis by human B cells is reported. Using this model, it is demonstrated that polyclonal T cells and CD4+ *Dermatophagoides* spp. (house dust mite)-specific T-cell clones derived from an atopic, house dust mite (HDM)-allergic individual are able to support IgE synthesis by autologous B cells. The helper activity was interleukin-4 (IL-4) dependent as only cloned T cells expressing detectable mRNA for IL-4 were able to induce IgE synthesis without the addition of exogenous IL-4. Peripheral and cloned T cells reactive with HDM could also be identified from a non-atopic individual but neither population was able to support IgE production even in the presence of exogenous IL-4.

INTRODUCTION

Immunoglobulin E (IgE) is responsible for allergic sensitivity in atopic individuals (Ishizaka, 1984). Although it is evident that IgE synthesis is regulated in part by interleukin-4 (IL-4), which in the mouse is secreted by a particular subset of helper T cells (Howard & Paul, 1983; Coffman *et al.*, 1986; Mosmann & Coffman, 1987), basic differences between atopic and non-atopic individuals in the regulation of IgE synthesis have not been resolved. This has been hindered by the absence of suitable allergen-dependent systems for *in vitro* IgE production by autologous B cells (Leung & Geha, 1987). Much of the available information has been derived from the analysis of pathological variants associated with hyper-IgE production (Geha *et al.*, 1981; Buckley & Fiscus, 1975) or from the use of alloreactive (Lanzavecchia & Parodi, 1984; Umetsu *et al.*, 1985) and autoreactive (Leung, Young & Geha, 1986) T-cell clones. Furthermore, polyclonal B-cell activators have failed to induce IgE synthesis in peripheral blood B cells of both non-atopic and allergic donors, thereby failing to provide suitable models for the analysis of IgE regulation (Saryan, Leung & Geha, 1983; Fiser & Buckley, 1979).

Abbreviations: Bu, biological unit; *D. farinae*, *Dermatophagoides farinae*, IgE, immunoglobulin E; IL-4, interleukin-4; PBMC, Peripheral blood mononuclear cells; SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis.

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In this report, an *in vitro* model of IgE synthesis using autologous B cells is described for the most common mite species inducing allergy (*Dermatophagoides* spp.; house dust mite, HDM) and, using allergen-specific polyclonal T cells and clones from an atopic and a non-atopic individual, the differences in IgE regulation are investigated.

MATERIALS AND METHODS

Antigen preparations

Lyophilized extracts of *Dermatophagoides farinae* (*D. farinae*), *Dermatophagoides pteronyssinus* (*D. pteronyssinus*), *Parietaria judaica*, Timothy grass and mixed grass pollen were kindly provided by Pharmacia (Uppsala, Sweden).

Isolation of human T cell clones

A panel of house dust mite-specific CD4+ T-cell clones was isolated from the peripheral blood of an atopic individual with perennial rhinitis and a non-atopic healthy subject, using the limiting dilution cloning method described previously (O'Hehir *et al.*, 1987). For cloning, viable cells (0·3 cells/well) were resuspended in complete medium (RPMI-1640 supplemented with 2 mM L-glutamine, 100 IU/ml penicillin/streptomycin) containing 5% screened human A+ serum and plated in Microtest II trays together with irradiated (2500 rads) autologous peripheral blood mononuclear cells (PBMC; 1×10^6/ml), *D. farinae* (10^3 BU/ml) and interleukin-2 (IL-2; Lymphocult T, 10% v/v, Biotest Folex, Frankfurt, FRG). Before use in assay systems the clones were rested for 6–8 days after the last addition of filler cells and antigen.

Proliferation assays

Cloned T cells (5×10^4/ml) were stimulated with *D. farinae* and mixed grass pollen at different concentrations in the presence of autologous irradiated PBMC ($1{\cdot}25 \times 10^5$/ml). After 60 hr incubation, tritiated methyl thymidine ([^{3}H]TdR; 1 μCi/well, Amersham International, Amersham, Bucks) was added and the culture harvested onto glass-fibre filters 8–16 hr later. Proliferation, as correlated with [^{3}H]TdR incorporation, was measured by liquid scintillation spectroscopy. For polyclonal T-cell responses, PBMC ($2{\cdot}5 \times 10^5$/ml) were stimulated with antigen for 6 days. The results are expressed as mean counts per minute (c.p.m.) for triplicate cultures.

Culture conditions for in vitro *antibody production*

Erythrocyte rosette negative cells (E^-) containing B cells and monocytes (< 2% contaminating E^+ cells) were isolated using aminoethylisothiouronium bromide hydrobromide (AET)-treated sheep erythrocytes and centrifugation on Percoll (Pharmacia) gradients (Lamb *et al.*, 1983). Cloned T cells ($2{\cdot}5 \times 10^5$/ml) or E^+ cells ($1{\cdot}5 \times 10^6$/ml) were cultured with *D. farinae* or mixed grass pollen (5×10^3 BU/ml) and autologous E^- cells ($2{\cdot}5 \times 10^5$/ml) in complete medium containing 10% fetal calf serum (FCS), with or without the addition of recombinant IL-4 (100 U/ml; Genzyme, Boston, MA). The supernatants were harvested at 10–12 days and the total levels of IgE and IgG determined by enzyme-linked immunosorbent assays (ELISA).

Determination of immunoglobulin synthesis

Total IgE and IgG were measured using solid-phase ELISA as described in detail elsewhere (Larche *et al.*, 1988). Briefly, for IgG, plates were coated with goat anti-human IgG (1 : 500; Miles Scientific, Slough, Bucks), blocked with 1% bovine serum albumin (BSA) and then incubated with the test samples. For detection of IgG, alkaline phosphatase-labelled goat anti-human IgG (1 : 500 dilution; Sigma, Poole, Dorset) conjugate was used and absorbance measured at 405 nm. For IgE, mouse anti-human IgE monoclonal antibody (1 : 500; Serotec, Kidlington, Oxon) was used to coat plates and biotinylated goat anti-human IgE (1 : 250; Vector, Peterborough, Cambs) was used as a second antibody followed by streptavidin biotinylated–horseradish peroxidase conjugate. Absorbance was measured at 450 nm. For both IgG and IgE assays standard curves were performed. The limits of detection were 8–1000 ng/ml and 50–12,000 pg/ml for IgG and IgE, respectively. IgG or IgM at concentrations of 50 μg/well were not detected in the IgE assay.

Dot blot analysis of T-cell clones

T-cell clones were activated on solid-phase anti-CD3 antibody (OKT3; Ortho Pharmaceutical Corp., Raritan, NJ) for 6 hr in the presence of 5% IL-2, collected, washed and resuspended in phosphate-buffered saline (PBS) containing cycloheximide (50 μg/ml) and vanadyl ribonucleoside complex (10 mM). Selective mRNA immobilization on the nylon membrane (Hybond-N, Amersham) was as described elsewhere (Bresser, Doering & Gillespie, 1983). Briefly, cells were lysed using pronase digestion followed by detergent treatment; the lysate was saturated with sodium iodide and put onto the membrane. After washing, the membrane was exposed to UV light before storage. Prehybridization and hybridization were carried out at 42° in 50% formamide and 6 × SSC (20 × SSC = 3 M NaCl + 0·3 M Na citrate). Human IL-2 cDNA (Taniguchi *et al.*, 1983) was labelled by random priming using a DNA-labelling kit (Boehringer Mannheim, FRG), and for IL-4 two partially overlapping reverse and complementary oligonucleotides (Yokota *et al.*, 1986)

(i) 5′-GTGCGACTGCACAGCAGTTCCACAGGCACA-3′
(ii) 3′-GTGTCCGTGTTCGTCGACTAGGCTAAGGAC-5′

were annealed and filled in using ^{32}PdCTP (Amersham) and three unlabelled dNTPs in the presence of Klenow enzyme.

Autoradiography from immunoblots of D. farinae

Mite body extract of *D. farinae* was separated by SDS-PAGE and transferred to nitrocellulose. The strips were probed with sera from the mite-allergic clone donor and from the non-allergic clone donor and autoradiographed to detect binding of IgE as described previously (O'Hehir *et al.*, 1987). Briefly, after blocking non-specific binding with 2% human serum albumin in Tris buffer (pH 7·5), dilutions of the sera (1 : 2 and 1 : 5) were allowed to react over the nitrocellulose sheets for 16 hr. After washing, they were incubated for a further 16 hr with ^{125}I anti-IgE (Pharmacia; 100 μl/approximately 10^4 c.p.m.). The washed, dried sheets were exposed to X-OMAT L Kodak film with Du Pont Quanta III intensifying screens at −70° for 48 hr and then developed.

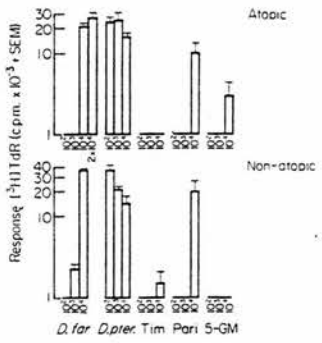

Figure 1. Antigen specificity of PBMC from an atopic and a non-atopic individual. PBMC ($2{\cdot}5 \times 10^5$/ml) were cultured with a panel of soluble aeroallergens at different concentrations. Proliferation was determined by [^{3}H]TdR incorporation after 6 days. Background responses of PBMC to medium in the absence of antigen were less than 115 c.p.m.

RESULTS

Antigen specificity of polyclonal and monoclonal T cells isolated from an atopic and a non-atopic individual

At the polyclonal level, the response of peripheral T cells from an atopic individual with perennial rhinitis and a non-atopic donor to *D. farinae* extract was comparable (Fig. 1). T cells from both individuals proliferated in a dose-dependent manner when stimulated with soluble *D. farinae* extract. Furthermore, proliferative responses were observed to the closely related member of the same genus, *D. pteronyssinus*, and to *Parietaria judaica* compared with the medium control.

Cloned T cells derived from the same individuals (clones DE26 and DE9 from the atopic subject and clone DF1 from the

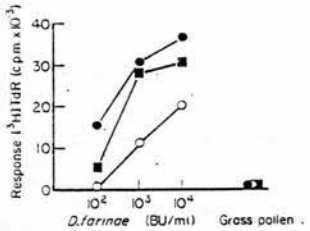

Figure 2. Cloned T cells (5×10^4/ml) were cultured with *D. farinae* and mixed grass pollen over a concentration range in the presence of autologous, irradiated PBMC ($1{\cdot}25 \times 10^5$/ml). Proliferation was determined by [^{3}H]TdR incoporation at 72 hr. Dose–response curves for *D. farinae* are shown: DE26 (open circles), DE9 (closed circles) and DF1 (closed squares). Only the maximum proliferative response to mixed grass pollen is plotted. Background responses to accessory cells in the absence of antigen were less than 275 c.p.m. and in all cases the percentage SEM was less than 25%.

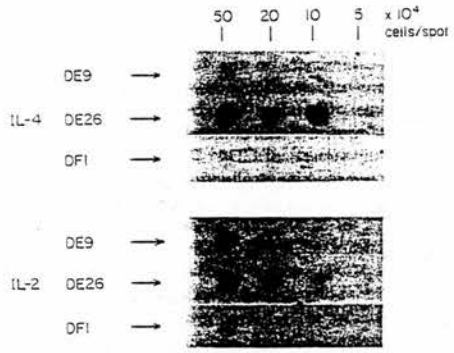

Figure 3. Dot blot analysis of mRNA for IL-2 and IL-4 in T-cell clones. Cloned T lymphocytes, activated for 6 hr with solid-phase anti-CD3, were probed using human IL-2 as cDNA and IL-4 oligonuclotides. Comparable mRNA for actin was present in all clones.

non-atopic subject) proliferated strongly in a dose-dependent fashion in response to the inducing allergen, *D. farinae*, but failed to recognize the unrelated allergen, mixed grass pollen (Fig. 2).

Dot blot analysis of mRNA for IL-2 and IL-4 in T-cell clones

The levels of IL-2- and IL-4-specific mRNA were compared for the T-cell clones DE9 and DE26 from the atopic and DF1 from the non-atopic individual 6 hr after activation with insolubilized anti-CD3 antibody (OKT3). Dot blot analysis of clones DE9 and DE26 revealed the presence of comparable levels of IL-2 transcripts (positive at 10×10^4 cell equivalents/spot: Fig. 3) whereas clone DF1 was positive at 50×10^4 cell equivalents/spot. DE26 also gave positive hybridization for IL-4 mRNA (10×10^4 cell equivalents/spot) as opposed to DE9, which was only weakly positive when mRNA from five-fold the number of cell-equivalents/spot was probed, and DF1, which had no detectable IL-4-specific mRNA.

Table 1. *D. farinae*-induced immunoglobulin synthesis by polyclonal and cloned T cells from an allergic individual

	T cells	E⁻	Antigen	IL-4	IgE pg/ml	IgG ng/ml
(a)	DE26	+	–	–	0	48
	DE26	+	*D. farinae*	–	252	565
	DE26	+	Grass pollen	–	0	30
	DE26	+	*D. farinae*	+	682	452
(b)	DE9	+	–	–	0	0
	DE9	+	*D. farinae*	–	0	73
	DE9	+	Grass pollen	–	0	0
	DE9	+	*D. farinae*	+	595	2843
(c)	E⁺	+	*D. farinae*	–	90	12
	–	+	*D. farinae*	–	0	0
	–	+	Grass pollen	–	0	0
	–	+	*D. farinae*	+	41	11
	E⁺	+	*D. farinae*	+	245	1335
	E⁺	+	–	+	40	0

T-cell clones DE26 (a) and DE9 (b) were isolated from the same atopic individual. IgE and IgG synthesis by E⁻ cells (c) cultured with IL-4 and antigen without added T cells are shown. Polyclonal T-cell help provided by E⁺ cells (c) was also determined. Background IgG and IgE synthesis by E⁻ cells cultured with antigen have been subtracted from the test groups. These were as follows: For (a), IgG ≤ 15 ng/ml, IgE ≤ 98 pg/ml; (b), IgG ≤ 15 ng/ml, IgE ≤ 90 pg/ml; (c), IgG ≤ 14 ng/ml, IgE ≤ 47 pg/ml.

Ability of *Dermatophagoides* spp.-specific T-cell clones to support IgE and IgG synthesis *in vitro*

Clone DE26 isolated from the atopic subject with detectable mRNA levels for IL-4 was able to collaborate with autologous E⁻ cells in the presence of the specific *D. farinae* allergen to support IgE synthesis. This was enhanced three-fold by the addition of exogenous IL-4 at the initiation of cultures (Table 1a). Clone DE9 with barely detectable IL-4 transcripts helped IgE synthesis only when exogenous IL-4 was added to the cultures (Table 1b). Similarly, allergen-dependent IgG synthesis mediated by T-cell clones DE26 and DE9 was also enhanced in the presence of exogenous IL-4. The failure of the autologous E⁻ cells cultured with antigen and IL-4 to stimulate marked IgE synthesis demonstrated the requirement for T cells in the system (Table 1c). The presence of antigen was required since E⁺ and E⁻ cells co-cultured with IL-4 were unable to induce IgE synthesis. Peripheral T cells (E⁺) from the atopic donor when cultured with autologous E⁻ cells and *D. farinae* induced the synthesis of IgE that was enhanced by the addition of IL-4 (Table 1c).

Cloned CD4⁺ T cells (DF1) from the non-atopic donor, which had no detectable mRNA for IL-4 (Fig. 3), were unable to induce IgE synthesis even in the presence of exogenous IL-4 (Table 2a), although able to support IgG secretion. Total IgG synthesis was also enhanced by the additional IL-4 added to the cultures. Similarly, stimulation of E⁻ cells from the non-atopic individual with allergen and peripheral T cells failed to induce

Table 2. *D. farinae*-induced immunoglobulin synthesis by polyclonal and cloned T cells from a non-atopic individual

	T cells	E⁻	Antigen	IL-4	IgE pg/ml	IgG ng/ml
(a)	DF1	+	–	–	0	0
	DF1	+	*D. farinae*	–	0	290
	DF1	+	Grass pollen	–	0	0
	DF1	+	*D. farinae*	+	0	420
(b)	E⁺	+	–	–	0	0
	E⁺	+	*D. farinae*	–	0	122
	–	+	*D. farinae*	+	20	190
	E⁺	+	–	+	40	200
	E⁺	+	*D. farinae*	+	55	360
(c)	E⁺ allo	+	–	–	93	26
	E⁺ allo	+	–	+	575	2385

T-cell clone DF1 (A) was isolated from a healthy, non-atopic individual. IgE and IgG synthesis by E⁻ cells cultured with IL-4 and antigen without added T cells is shown (b). Polyclonal T cell help provided by E⁺ cells (b) and cloned allogeneic E⁺ cells (c) are shown. Background immunoglobulin synthesis by E⁻ cells cultured with antigen have been subtracted from the test groups. These were as follows: For (a), IgG ≤ 70 ng/ml, IgE ≤ 190 pg/ml; for (b) and (c), IgG ≤ 15 ng/ml, IgE ≤ 94 pg/ml.

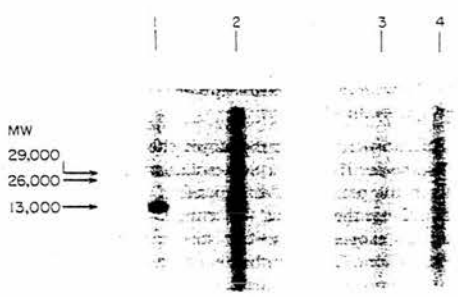

Figure 4. Autoradiographs of Western blots showing the IgE response of sera to *D. farinae*. Mite body extract of *D. farinae* was separated by SDS-PAGE and transferred to nitrocellulose. The strips were probed with sera from the mite allergic clone donor (1 : 2) dilution, lane 1; 1 : 5 dilution, lane 2) and from the non-atopic clone donor (1 : 2 dilution, lane 3; 1 : 5 dilution, lane 4) and autoradiographed to detect binding of IgE.

IgE secretion, although IgG was produced, albeit at low amounts (Table 2b). The addition of exogenous IL-4 to these cultures did not markedly enhance IgE synthesis above that observed in the controls (Table 2b).

The stimulation of B-cell enriched populations from the non-atopic subject with cloned allogeneic T cells confirmed their capacity for the synthesis of IgE (Table 2c). This response was strongly enhanced in the presence of exogenous IL-4 for both IgE and IgG synthesis.

Reactivity pattern of specific IgE to immunoblots of *D. farinae*

Autoradiographs using serum from the allergic donor of clones DE9 and DE26 showed strong IgE binding to the *D. farinae* immunoblots (Fig. 4). A distinct band was evident at 13,000 MW, which corresponds to the *Der f* II allergen of *D. farinae* that has recently been recognized as a major allergen. Fainter bands were also evident at 26,000 and 29,000 MW, corresponding to the *Der f* I and *Der f* III allergens.

The autoradiographs using serum from the non-atopic clone donor showed no evidence of specific IgE directed at any of the determinants of *D. farinae*, consistent with the lack of allergic symptoms, negative prick skin tests and negative radioallergosorbent testing (RAST; data not shown).

DISCUSSION

The inability to isolate antigen-specific T cells combined with the absence of an allergen-dependent *in vitro* model of antibody synthesis has restricted the investigation of differences in T-cell regulation of IgE induction in atopic and non-atopic individuals. In this report it is demonstrated that the T-cell repertoires of an atopic and a normal subject both include cells capable of recognizing house dust mite allergen, and that HDM-specific T-cell clones can be isolated from each donor. Furthermore, differences in the functional activity of these T-cell clones and polyclonal E⁺ cells were examined in an *in vitro* model of allergen-dependent IgE synthesis.

Analysis of murine T-cell clones suggests that there are two non-overlapping subsets of CD4⁺ T cells (Th1 and Th2) that differ in their lymphokine production and functional activities (Mosmann & Coffman, 1987). The Th2 cells, which produce IL-4 but not IL-2 or IFN-γ, are potent helpers for B cell IgE synthesis (Howard & Paul, 1983; Coffman *et al.*, 1986). Therefore, the HDM-specific T-cell clones described here were analysed for their ability both to produce IL-4, as determined by dot blot analysis for mRNA, and to support IgE synthesis. The T-cell clones from the atopic individual differed markedly in their levels of IL-4 transcripts, whereas no mRNA for IL-4 could be detected in the cloned T cells (DF1) derived from the non-atopic subject. In contrast, all the clones had detectable levels of IL-2 transcripts. Thus, the dichotomy of lymphokine production observed for murine T-cell clones appears not to apply so broadly to human T-cell clones. From the heterogeneity of T cells as regards their ability to secrete lymphokines (Del Prete *et al.*, 1988), caution must be exercised in extrapolating from the analysis of these clones to the intact T-cell repertoire of the individuals tested in this study. However, the functional characteristics observed at the polyclonal T-cell level would support these observations.

Investigation of the functional activity of the HDM-specific T-cell clones revealed that clone DE26, with marked IL-4 mRNA levels when co-cultured with autologous E⁻ cells, and *D. farinae* induced IgE synthesis that was enhanced by the addition of exogenous IL-4. In contrast, clone DE9, also isolated from the atopic individual but with minimal levels of IL-4 transcripts, supported IgE synthesis only in the presence of exogenous IL-4. The T-cell dependence of IgE induction was demonstrated by the failure of E⁻ cells to synthesize IgE when cultured with only IL-4 and *D. farinae*. Similarly, the necessity for antigen is demonstrated as E⁺ and E⁻ cells co-cultured with IL-4 alone failed to induce IgE synthesis, and this suggests that

antigen rather than IL-4 activates the T cells to provide B-cell help. Further support for this is that the T-cell clones are unresponsive to IL-4 stimulation (data not shown). The observation that peripheral E^+ cells from the atopic individual were capable of supporting allergen-dependent IgE synthesis implies that the T-cell clones were not unique cells but representative of those in the polyclonal population. The relatively low levels of IgE induced by polyclonal T cells reflects the low numbers of HDM-immune T cells circulating in peripheral blood (Halvorsen, Bosnes & Thorsby, 1986). These findings are consistent with the concept that B cells from atopic individuals are able to synthesize IgE when the appropriate T-cell help is provided.

In contrast, both polyclonal and cloned $CD4^+$ T cells reactive with HDM from the non-allergic subject were unable to induce allergen-dependent IgE synthesis even following the addition of exogenous IL-4. The presence and absence of *D. farinae*-specific IgE in the sera of the atopic and non-atopic individuals, respectively, was confirmed by immunoblotting and autoradiography and suggests that the *in vitro* findings reflect the *in vivo* status of these subjects. The stimulation of B cells from the non-atopic donor with cloned allogeneic T cells confirmed their capacity to synthesize IgE. It is well documented that non-atopic individuals are capable of generating an IgE response following parasitic infection (Nutman *et al.*, 1985) and allostimulation (Umetsu *et al.*, 1985). The failure of clone DF1, or of the peripheral E^+ cells from the non-atopic subject, to induce IgE synthesis cannot be explained solely by its inability to secrete IL-4 for the addition of exogenous IL-4 did not overcome the defect. Additional regulatory mechanisms may be required (Maggi *et al.*, 1988) or there may be a defect in the IL-4 receptor. It has been suggested that the inability of T cells lacking IL-4 to support IgE synthesis is partly due to the production of IFN-γ (Mosmann & Coffman, 1987; Coffman & Carty, 1986; Snapper & Paul, 1987). Nevertheless, in the presence of anti-IFN-γ antibody and IL-4, clone DF1 was unable to provide appropriate help although marked enhancement of IgE synthesis was observed under similar culture conditions with the clone DE9 (data not shown). Furthermore, the observation that cloned $CD4^+$ HDM-specific T cells from the non-atopic individual fail to support IgE synthesis contradicts the hypothesis that failure of IgE production in non-allergic subjects results solely from inhibition by regulatory ($CD8^+$ suppressor) T cells. Nevertheless, it must be emphasized that population studies are required to resolve differences unequivocally in the regulatory mechanisms of both atopic and non-atopic individuals in their responses to allergen.

The absence of allergen-triggered IgE in the non-atopic system may result from the absence of specific B cells which can act as highly efficient antigen-presenting cells concentrating antigen via Ig receptors (Lanzavecchia, 1985) which are already clonally expanded in the atopic individual. Alternatively, the failure to synthesize IgE could be due to a defect in isotype switching (Cebra, Komisar & Schweitzer, 1984; Radbruch *et al.*, 1986; Gearhart, Sigal & Klinman, 1975) in the non-atopic subject. However, the induction of IgE synthesis by allostimulation makes this explanation unlikely. Interestingly, both subjects were able to synthesize IgG in response to *D. farinae* stimulation.

If the T-cell clones studied here recognize different determinants within the allergen and the T-cell recognition of a particular epitope within a protein can influence immune function (Adorini *et al.*, 1977) then this could also offer an explanation for the inability of DF1 to induce IgE synthesis. This is supported by the observation that only the peripheral T cells of HDM allergic subjects respond to affinity-purified *Der p* I allergen (Rawle *et al.*, 1984). Furthermore, as T-cell antigen recognition depends upon the binding of peptide antigen to MHC proteins, the haplotype of the individuals will influence the epitopes recognized and the immune response induced (Rothbard *et al.*, 1988). Indeed, this is supported by the observation that immune responsiveness to *Amb a* VI in allergic individuals, as assessed by the presence of specific IgE in the serum, is HLA-DR5 associated (Marsh *et al.*, 1987).

The experimental model described here has allowed analysis of qualitative differences in helper activity for allergen-dependent *in vitro* IgE synthesis mediated by *Dermatophagoides spp.*-specific polyclonal and monoclonal T cells derived from an atopic and a non-atopic individual. Knowledge of mechanisms involved in such IgE regulation is required to facilitate the development of effective therapeutic regimens for allergic diseases.

ACKNOWLEDGMENTS

Dr Robyn O'Hehir is the recipient of a Sir Robert Menzies Medical Scholarship. This work was supported by the Menzies Trust and the Clinical Research Committee of the National Heart and Chest Hospitals, London. The authors are grateful to Drs I. Goldsmith and T. Taniguchi for the gifts of the cDNA probes and to the Department of Microbiology, National Heart & Lung Institute, Brompton Hospital, London, for the generous use of facilities. We thank Drs M. D. Chapman, A. Cooke, R. Lechler and M. Owen for helpful scientific discussion.

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Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 8214–8218, September 1993
Immunology

In vivo clonal dominance and limited T-cell receptor usage in human CD4$^+$ T-cell recognition of house dust mite allergens

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Communicated by J. L. Gowans, June 7, 1993 (received for review March 4, 1993)

ABSTRACT **Sensitivity to house dust mite antigens in atopic individuals is a major cause of allergic diseases, ranging from asthma to rhinitis and dermatitis. We have studied the T-cell receptor (TCR) usage of house-dust-mite-specific CD4$^+$ T-cell clones isolated from an atopic individual, by using the anchored polymerase chain reaction, and have analyzed the peripheral TCR repertoire of the same individual. Several T-cell clones had identical TCRs at the sequence level, despite the fact that they had been independently isolated, in some cases, in different years. These data suggest the presence *in vivo* of long-lived T-cell clones. We have also shown that junctional sequences identical to these clones are present in peripheral blood T cells taken 6 years after the isolation of the T-cell clones. The analysis of TCR genes used by the panel of clones reveals oligoclonality, with the variable (V) region gene segments Vα8 and Vβ3 being dominant, although there is minimal conservation of junctional sequences. The results have implications for understanding the TCR recognition of an environmental aeroallergen and the life span of T-cell clones *in vivo* during a chronic immune response.**

The production of specific IgE by atopic individuals after exposure to common environmental aeroallergens represents an aberrant and potentially pathogenic immune response. One-third of the population is atopic, whereas allergic diseases affect ≈10% of the population. CD4$^+$ T lymphocytes play a central role in the regulation of IgE production (1, 2). Human house-dust-mite (HDM)-specific CD4$^+$ T-cell clones isolated from atopic individuals show an "interleukin (IL) 4-dominant" (Th2) functional phenotype (3, 4) and promote specific IgE synthesis *in vitro* from autologous B cells (3). In contrast, HDM-specific T-cell clones from nonatopic individuals express an "interferon γ-dominant" (Th1) phenotype and fail to support IgE production *in vitro*. The two major species of HDM, *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*, produce ubiquitous allergens that are derived from the fecal pellets and bodies of the mites. There are at least four major groups of HDM antigens (groups I–IV). The cloning and sequencing of some of these allergens (5) have facilitated the study of their major T-cell epitopes; however, to our knowledge, the T-cell receptor (TCR) gene segments that recognize these antigens have not been previously investigated.

The TCR $\alpha\beta$ heterodimer confers antigen specificity through the use of different variable [α chain V region (Vα) and β chain V region (Vβ)], diversity (Dβ), and joining (Jα and Jβ) gene segments that undergo somatic rearrangement during thymocyte development (6). The insertion or deletion of junctional nucleotides creates further diversity (for review, see ref. 7). Studies of TCRs recognizing defined peptide–major histocompatibility complex (MHC) complexes have shown restriction of V gene usage and/or conservation of junctional sequences (8–10). Other groups have demonstrated oligoclonality of V gene usage by T cells isolated directly from a disease site, where the antigen specificity is unknown (11, 12). There have been few detailed studies of TCR sequences from human T cells whose central role in a chronic immune response is well documented and, to our knowledge, no such previous studies in the field of allergy.

We have analyzed the TCR sequences of a panel of HDM-specific T-cell clones from an individual with perennial rhinitis, using the anchored PCR.[¶] These clones are heterogeneous in both antigen and HLA class II restriction specificities, yet analysis of TCR gene usage indicates limited diversity. The unexpected finding of identical TCR sequences in clones isolated in different years led us to screen peripheral blood lymphocytes (PBLs) isolated 6 years later for the same junctional sequences.

MATERIALS AND METHODS

Antigens. Lyophilized unfractionated extracts of *D. pteronyssinus* and *D. farinae* HDM were generously provided by Bencard (Brentford, Middlesex, U.K.) and H. Lowenstein, (ALK Laboratories, Copenhagen).

PBL and T-Cell Clones. PBLs were isolated by Ficoll density gradient centrifugation. HDM-specific T-cell clones were isolated from an atopic donor with perennial rhinitis as described (13). Serological typing showed the haplotype of this donor to be A2, 24(9); B50(21), 27; DR11(5), 7; DR52, 53; and DQ2, 7. Briefly, PBLs were stimulated for 7 days with unfractionated *D. farinae* extract, and activated T cells were then cloned by limiting dilution (0.3 cells per well) in the presence of specific antigen, irradiated autologous PBLs as accessory cells, and IL-2. To determine MHC restriction specificity, T-cell clones (1×10^5 cells per ml) were stimulated with HDM (1–30 μg/ml) in the presence of an equal number of irradiated (2500 rads; 1 rad = 0.01 Gy) autologous PBLs or mitomycin C-treated murine fibroblasts expressing HLA-D region gene products as antigen-presenting cells. Murine fibroblasts (DAP3), transfected with the following HLA-D region genes, were used: *DRB1*0101* (from J. Trowsdale, ICRF), *DRB1*1201* and *DRB4*0101* (from S. Rosen-Bronson, Georgetown University, Washington, DC), and *DRB3*0101* and *DRB3*0201* (from B. Mach, University of Geneva, Geneva). After a 60-hr incubation, the cultures were pulse-labeled with [^{3}H]thymidine (1 μCi per well; 1 Ci = 37 GBq; Amersham) and harvested 8–16 hr later. Proliferation

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Abbreviations: TCR, T-cell receptor; V, variable; D, diversity; C, constant; J, joining; IL, interleukin; MHC, major histocompatibility complex; PBL, peripheral blood lymphocyte; Vβ, Cα, etc., β chain V region, α chain C region, etc.; HDM, house dust mite.

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[¶]The sequences reported in this paper have been deposited in the GenBank data base (accession nos. Z22965–7 and Z23039–47).

as correlated with [^{3}H]thymidine incorporation was determined by liquid scintillation spectroscopy.

Isolation of RNA, cDNA Synthesis, and PCR. Total RNA was isolated from T-cell clones and PBLs by the acid guanidinium thiocyanate/phenol method (14). Total RNA (5 μg) was used in first-strand cDNA synthesis, using a $dT_{(12-18)}$ primer and avian myeloblastosis virus reverse transcriptase. The products were precipitated three times using 2 M ammonium acetate and 3 vol of ethanol and then tailed with dGTP by using terminal deoxynucleotidyltransferase. Five percent of the cDNA product was used as template in the PCR with a 5′ polydeoxycytosine primer (5′-CTATCTAGAGAGCTCGCGGCCGCCCCCCCCCCCC) and a 3′ primer for the β chain constant region (Cβ) (5′-CGCGAATTCAGATCTCTGCTTCTGATG), or the α chain constant region (Cα) (5′-TGACCGCAGTCGACAGACTTGTCACTGGATT). Reaction conditions were as follows: one cycle of 94°C for 5 min, 55°C for 1 min, and 72°C for 5 min, followed by 29 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, with a final extension period of 10 min. These reactions produced specific products of 600–650 bp.

Cloning and Sequencing of Amplified TCR Gene Products. After treatment with proteinase K, the amplified products were digested with *Not* I and *Bgl* II (TCRβ chain products) or *Not* I and *Sal* I (TCRα chain products) and cloned into a modified form of the M13mp18 bacteriophage vector (kindly supplied by P. Moss and J. Bell, Oxford). DNA sequencing was by the dideoxynucleotide chain-termination method (15), using T7 DNA polymerase. For T-cell clones, between 10 and 20 M13 clones from each amplification were sequenced. From the products of PBL cDNA amplification, 50 TCRα and 50 TCRβ in-frame sequences were determined and assigned to previously published V gene families (7, 16–23). Out-of-frame or aberrant rearrangements were not included in the 50 sequences but were noted.

Oligonucleotide Probing of PBL Transcripts. For the screening of PBLs for TCR sequences that were identical to those of the T-cell clones, venous blood was taken 6 years after the original cloning work, and cDNA was prepared. PCR was performed using the 3′ Cβ primer as above and a 5′ Vβ primer for Vβ21 (5′-TGCTGGGCGGCCGCCTGTCTCCTGGGAG) or Vβ6 (5′-GGATGTAGAGCTCAGGTGTGATCCAATTTCAG) families with conditions as follows: 94°C for 5 min, followed by 29 cycles of 94°C for 1 min, 63°C for 1 min, and 72°C for 2 min. PCR products were digested, cloned into modified M13mp18, and transferred to Hybond-N+ nylon filters. Oligonucleotides were labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP (5000 Ci/mmol, Amersham). Filters were probed with oligonucleotides complementary to the N region of the clones of interest, (DH15N, 5′-CTCTCCTCGACTGCTCCCGCTAG; DD11N, 5′-GGCTGGTTTCCCCCCCTATCTAA), and internal Vβ-specific oligonucleotides (Vβ6.7a, 5′-GAGCTCAGGTGTGATCCAATTTCA; Vβ 21.5, 5′-TGTGGCTTTTTGGTGCAATCCTAT). Membranes were prehybridized in 6× standard saline citrate (SSC)/5× Denhardt's solution/0.1% SDS and hybridized at 58°C for 16 hr. They were then washed at 45°C in 3× SSC/0.1% SDS for 10 min before exposure to x-ray film for 4 hr at −70°C. Positive plaques were picked and the DNA sequences of their inserts were determined.

RESULTS

Recognition Patterns of T-Cell Clones. The antigen specificity of the panel of 10 T-cell clones was examined using *D. farinae*, the inducing antigen, and a closely related HDM species, *D. pteronyssinus*. Six of the clones were species specific to *D. farinae*, and the remaining T-cell clones were cross-reactive on *D. farinae* and *D. pteronyssinus* (Table 1). Further analysis of these cross-reactive clones showed that they were specific for the group I allergen (data not shown). The MHC class II restriction specificities of the T-cell clones, as determined using transfected murine fibroblasts expressing HLA-D region gene products, are shown in Table 1.

Table 1. Antigen specificity and MHC restriction of HDM-induced T-cell clones

Clone	Proliferation to		MHC restriction
	D. farinae	*D. pteronyssinus*	
DE41	+	−	DR52
DE47	+	−	DR52
DE12	+	−	DR52
DD11	+	−	DR52
DH15	+	+	DR11(5)
DE9	+	+	DR11(5)
DE5	+	−	DR53
DE26	+	+	DR52
DH12	+	+	DR11(5)
DE49	+	−	ND

ND, not determined.

TCRα and TCRβ Gene Rearrangements in T-Cell Clones. The Vα and Vβ regions used by the 10 T-cell clones analyzed are summarized in Table 2. The nucleotide and predicted protein sequences spanning the junctional V(D)J regions of the TCRα and TCRβ chains are shown in Fig. 1. Only one in-frame TCRβ rearrangement was detected in each of the T-cell clones. However, in several T-cell clones, two distinct in-frame TCRα sequences were detected. In each case, one TCRα transcript was always found at a much higher frequency than the other in the PCR amplification products. Therefore, it is most probable that the products of the major TCRα transcript are expressed in association with the TCRβ chain, at the cell surface. Six T-cell clones utilized unique TCR sequences, whereas four clones were found to be identical at the sequence level (including the sequence across the VDJ or VJ junctions) to others within the panel. Thus, DE12, DE41, and DE47 were identical to DD11, and DE9 was identical to DH15, although all clones had been isolated separately, and clones with different letters (DD, DE, or DH) originated from different blood samples. Specifically, clone DD11 was established in the year prior to the DE clones, whereas DE9 and DH15 were isolated 10 months apart.

Oligoclonality of V Gene Usage. A restricted number of TCR V genes were used by the panel of T-cell clones. Three clones, DH12, DE26, and DE49, used Vβ3.1, whereas members of the Vα8 family were used by three clones, DD11 (itself identical to three other clones), DH12, and DH15 (itself identical to one other clone) (Table 2). The Vβ sequence of DD11 (and DE12, DE41, and DE47) is identical at the protein level to the cDNA clone VbIW10 (22), where it is named Vβ21.5, and differs by only 1 nt, which is silent at the protein level, from a sequence (cDNA clone IGRb01) classified

Table 2. Summary of TCR V gene segments used by the panel of T-cell clones

Clone(s)	TCRβ				TCRα	
	V	D	J	C	V	J
DE49	3.1	1	1.2	1	15.1	AB19
DE26	3.1	2	2.5	2	1.2	JαG
DH12	3.1	1	1.1	1	8.2	IGRJa04
DD11, DE12 DE41, DE47	21.5	1	1.5	1	8.1	AB22
DH15, DE9	6.7a	2	2.7	2	8.1	JαC
DE5	9.1	1	1.4	1	Vδ5	JαN

TCR α and β sequences were assigned according to previously described families, by using refs. 7, 16, 18, 21, and 22 for TCRβ chains and refs. 7, 17, 20, 24, and 25 for TCRα chains.

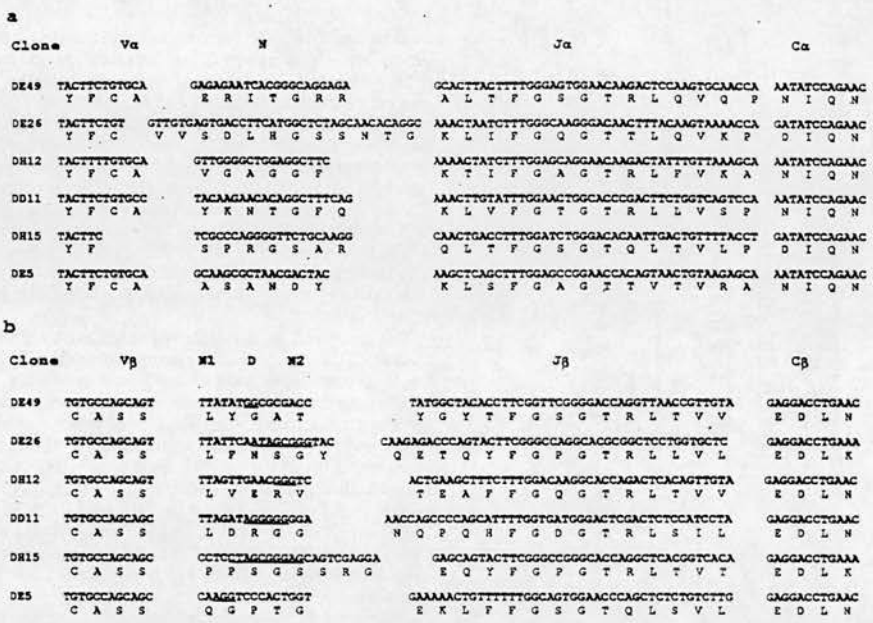

FIG. 1. Sequence analysis of HDM-reactive T-cell clones. DNA sequences encoding TCRα (*a*) and TCRβ (*b*) chains spanning the junctional regions are shown. Predicted amino acid sequences are also given. Spaces have been introduced to allow alignment of conserved sequences. The nucleotides contributed by germ-line Dβ sequences are underlined. Those T-cell clones with identical junctional sequences (i.e., DE9, identical to DH15; DE12, DE41, and DE47, all identical to DD11) are not shown.

within the Vβ21 family (19). The Vα segment of DE5 is classified as a Vδ gene (17) but has rearranged to a Jα and the Cα segment. This phenomenon is now well recognized (26, 27) and has been estimated to occur in ≈1% of all productive TCRα rearrangements (23). However, we believe this to be the first demonstration of the use of Vδ5 in a TCRα chain of a well-characterized human T-cell clone. There was no detectable conserved motif in the V(D)J junctional region of either the TCRα or TCRβ chains, although four of the six TCRβ chains have a leucine residue at position 96 (28), the first coded for by N region nucleotides.

Screening of PBLs for Persistent Clones. To study the possible *in vivo* life span of T-cell clones that appear to persist in this chronic response, venous blood was taken 6 years after the original isolation of these clones. cDNA was prepared and screened for the TCRβ sequences of interest by using a Vβ-specific family PCR and oligonucleotide probing. In both cases, sequences identical to the VDJ region of the clones were demonstrated in full-length in-frame transcripts from unstimulated PBLs (Fig. 2).

Peripheral Repertoire Studies. The peripheral blood TCR Vα and Vβ gene usage of the individual from whom the T-cell clones were established was analyzed by anchored PCR. The repertoire of this individual is shown in Fig. 3. Each of the 50 VαJ or VβDJ in-frame productive sequences was unique. The frequency of Vα and Vβ usage was in broad agreement with previously published repertoire studies, although there is considerable interindividual variation (29). Three TCRα chains used a Vδ gene segment. In addition to the productive TCR sequences, several sequences could not code for a functional TCR chain. Thus, 10% of Vα and 5% of Vβ rearrangements had no open reading frame for VDJ translation. The changes in translational reading frame were always found within the junctional regions, consistent with out-of-frame rearrangements, rather than errors in the PCR amplification. In addition, 5% of both TCRα and TCRβ transcripts contained only D and/or J sequences 5′ of Cα or Cβ or contained germ-line sequence 5′ to the C gene segments. PBLs from this individual were also stained with a panel of antibodies specific for Vβ gene products, within the CD3-, CD4-, and CD8-positive populations and analyzed by immunofluorescence. These data were in broad agreement with those obtained by PCR analysis (data not shown).

DISCUSSION

In this study, designed to investigate the diversity of TCR usage by CD4$^+$ T cells in atopic individuals sensitized with allergens of *Dermatophagoides* spp. (HDM), we have observed dominant expression of TCR Vβ3 and Vα8 genes. Furthermore, the TCR sequence data suggest the presence of dominant T-cell clones that are long lived *in vivo*, presumably maintained by chronic allergen exposure. The analysis of the HDM-specific CD4$^+$ T-cell response in atopic individuals has attracted considerable attention (13, 30). The results of such studies have established that T cells expressing an IL-4-dominant phenotype are predominantly activated. From our investigation of the antigen and HLA class II restriction specificity, it appears that even within one individual the HDM T-cell response is heterogeneous. Several epitopes derived from both the group I and II major allergens are known to be recognized by "disease-associated" T cells, namely, those that produce high levels of IL-4 and support the production of HDM-specific IgE. It has also been demonstrated that HLA-DRB1, -3, -4, and -5 gene products and DP class II molecules may restrict recognition of HDM-derived T-cell determinants (31).

It is remarkable, therefore, that a considerable degree of oligoclonality was observed in this panel of HDM-reactive clones. A further unexpected observation was that two

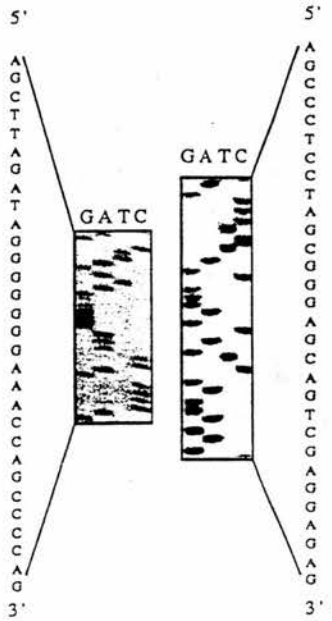

FIG. 2. Autoradiographs from sequencing gels of PBL TCRβ transcripts showing sequences corresponding to the N-region nucleotides of the DD11 (*Left*) and DH15 (*Right*) T-cell clones (for comparison of these sequences with those in the T-cell clones, see Fig. 1).

groups of T-cell clones used identical TCRs. Each of these groups must represent cells that originated from one precursor because the TCR sequences were identical even across the N regions. We have also demonstrated sequences identical to the TCRβ N regions in unstimulated PBLs from 6 years later. It is possible that different peripheral blood T cells possess TCRβ sequences identical at the nucleotide level even across the CDR3 junctional region but use different TCRα chains. However, we believe that these data provide strong evidence for the existence of the same long-lived clone over 6 years. This is to our knowledge the first report of such long-lived T-cell clones *in vivo*. One explanation for the existence of long-lived clones in this individual is that disease-specific T cells are constantly stimulated to divide by chronic exposure to HDM antigens. In this context, it is interesting that the CD45RO "memory" T-cell population has been shown to have a short intermitotic time *in vivo* (32).

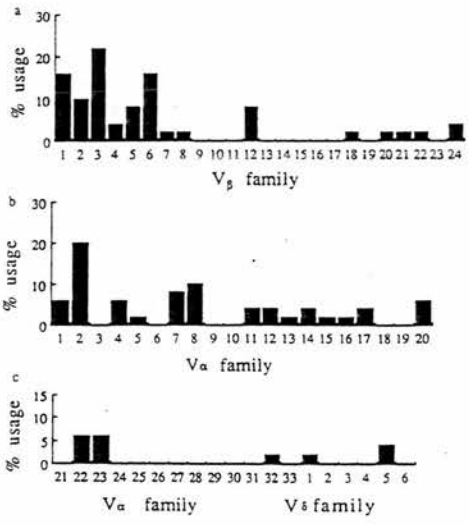

FIG. 3. Vβ and Vα repertoire analysis. The percent usage of Vβ1-24 (*a*), Vα1-20 (*b*), and Vα21-33 and Vδ1-6 (where a Vδ has rearranged to the Cα gene) (*c*) in 50 PBL TCRα and 50 TCRβ sequences analyzed is shown.

The anchored PCR has now been used by several groups to study TCR use by T-cell clones and lines and PBLs. It has the theoretical advantage of being able to amplify all possible TCR sequences equally, since the two primers are the same for any transcript. The results of our repertoire study are in many ways comparable to other studies, although the high representation of Vβ3 in PBL transcripts from this individual was unusual. In other reports, Vβ3 usage is generally between 1 and 6% of TCRβ in PBLs (ref. 29 and L.R.W., unpublished observations). Many factors could account for this, such as MHC or other genetic differences and environmental influences, all of which will have affected thymic selection.

The potential to inhibit allergic inflammation using components of specific antigen is the underlying goal of allergen-based desensitization. There is evidence to suggest the presence of dominant antigenic regions in the major allergens of HDM, but overall both the array of epitopes recognized and the number of restriction elements used in the T-cell response of atopic individuals are diverse. Therefore, while it has been reported that the administration of immunodominant T-cell epitopes, as peptides, *in vivo* can induce antigen-specific nonresponsiveness (33), peptide-based hyposensitization may be difficult to achieve for allergic responses to HDM. Furthermore, in some of the autoimmune diseases, the specificity of the "disease-associated" T-cell repertoire may shift as illustrated in murine experimental allergic encephalomyelitis (34). During the induction phase of disease, the T-cell response is directed toward an immunodominant NH_2-terminal region of myelin basic protein, but as the disease becomes chronic, additional determinants that are cryptic after the initial immunization then become immunogenic (34).

From TCR sequence analysis of cloned T cells and PBLs, we suggest that the *in vivo* T-cell response to HDM is dominated by, and limited to, a small number of long-lived T-cell clones, which use a restricted number of TCR gene segments. Therefore, even if responses to minor T-cell determinants are retained, inactivation of the dominant HDM-reactive T cells might allow a decrease in clinical symptoms. In the experimental allergic encephalomyelitis model, it has been reported that the "pathogenic" T cells predominantly express the Vβ8$^+$ TCR and immunization with CDR2 peptides derived from Vβ8 prevents disease (35). This would support the potential application of TCR-based therapy as an alternative approach for regulating HDM allergic immune responses.

We thank Drs. P. Moss and J. Bell for invaluable advice, discussions, and the gift of reagents, as well as for critical reading of the manuscript. We are grateful to Dr. P. Beverley for helpful comments on the manuscript and to Ms. V. Weller for preparing the manuscript. This work was supported by the Imperial Cancer Research Fund, the Medical Research Council, and the Wellcome Trust. R.E.O. is a Wellcome Senior Research Fellow.

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Analysis of human T cell responses to the group II allergen of *Dermatophagoides* species: Localization of major antigenic sites

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Background: *IgE antibodies reactive with the group II allergens of* Dermatophagoides *species (house dust mite [HDM]) are a major component of the allergic immune response in HDM-allergic atopic individuals.*

Methods: *Here we demonstrate, with the use of overlapping synthetic peptides of the group II allergen of* Dermatophagoides pteronyssinus *(Der p II), that polyclonal T cells isolated from the majority of atopic HDM-allergic individuals and healthy nonatopic control subjects respond to* Der p *II and that T-cell epitopes are present in all regions of the protein.*

Results: *From comparison of peptide-specific T-cell proliferation in both groups of individuals, it appears that together peptides 61-86 and 78-104 are the most frequently recognized (16 of 18 individuals). We also observed that nine of the 18 individuals responded to T-cell epitopes in the region 11-50, and with* Der p *II–reactive T-cell clones, three distinct T-cell epitopes were mapped within the sequence 11-35. Also, with the use of T-cell clones, two additional epitopes were identified at residues 81-96 and 91-101.*

Conclusions: *These results suggest that T-cell epitopes located in these regions (11-50 and 61-104) are immunodominant. The value of this information in the potential application of* Der p *II peptides to desensitize HDM allergic responses is discussed. (J Allergy Clin Immunol 1993;92:105-13.)*

Key words: Peptides, T cells, Dermatophagoides species, house dust mite, Der p II, densensitization

In approximately 15% of the population,[1] T-cell recognition of proteins derived from common environmental aeroallergens may result in both the induction of specific IgE synthesis and the activation of polymorphonuclear granulocytes.

Abbreviations used:
APCs: Antigen presenting cells
HDM: House dust mite
IL-2: Interleukin-2
PBMCs: Peripheral blood mononuclear cells

Together these two effector pathways contribute to the allergic immune response.[2-5] The clinical symptoms that result from the activation of these effector pathways range from extrinsic asthma and allergic rhinitis to atopic dermatitis. Evidence accumulated from clinical studies has implicated proteins from the house dust mite (HDM) *Dermatophagoides* species (*D. pteronyssinus* and *D. farinae*) as a primary cause of respiratory allergy.[6, 7]

Multiple antigens have been identified in extracts of HDM that bind IgE antibodies present in the sera of HDM-allergic patients[8]; nevertheless,

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Supported by grants from the Wellcome Trust and the Medical Research Council, England.

R. E. O'Hehir is the recipient of a Wellcome Senior Research Fellowship in Clinical Sciences.

Received for publication Aug. 25, 1992; revised Jan. 6, 1993; accepted for publication Jan. 27, 1993.

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0091-6749/93 $1.00 + .10 1/1/46030

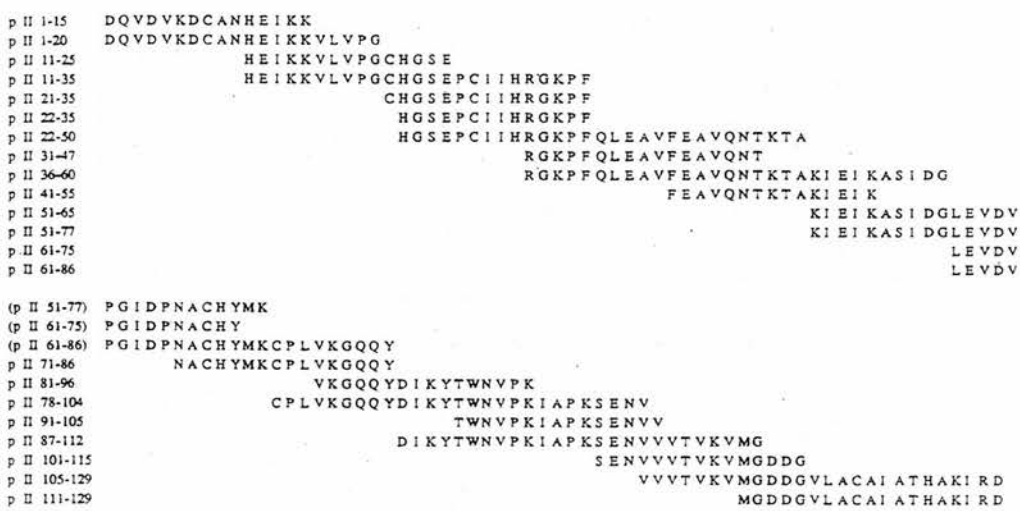

FIG. 1. Amino acid sequences of overlapping synthetic peptides derived from *Der p* II. Residues are indicated with the single letter code.

the major component of the humoral response appears to be directed against four major allergic proteins (groups I to IV).[8-12] Initially, although interest focused on the analysis of group I allergen-specific IgE, the importance of the group II allergens has now been substantiated by several independent studies, and it appears that the anti-group II (*Der p* II) reactive antibodies may account for between 40% and 88% of the HDM-specific IgE antibody response in HDM-allergic individuals.[8, 11] Indeed in one report it was demonstrated that in the serum of 92% of the HDM-allergic patients investigated, IgE antibodies reactive with *Der f* II could be detected, whereas anti-*Der f* I and *Der f* III antibodies were present in the sera of only 78% and 16% of the patients, respectively.[10]

It is well established that CD4+ T-cell recognition of allergen is central to the induction of specific allergic immune responses.[2-5] However, for many aeroallergens, including HDM, detailed information on the localization and frequency of T-cell epitopes within these proteins is limited. The identification of dominant T-cell determinants in specific allergens is relevant to understanding the functional role of antigen-specific T cells in the development of allergic inflammation and a critical initial step in the development of allergen-based immunotherapy.[13, 14] There is evidence indicating that all of the individual group-specific proteins of HDM are antigenic for T cells at the polyclonal level.[4] However, on the basis of the initial serologic data, analysis of the antigen specificity of the HDM-reactive T cells has focused primarily on the response of atopic individuals to group I allergen prepared by electrophoresis and gel filtration.[15] Similar to antibody responses, it is now apparent from specificity studies with T-cell clones,[16] and more recently polyclonal populations,[17] that a major component of the peripheral T-cell repertoire is reactive with the group II allergens. The complementary DNA sequences that encode both the group I and II genes have been cloned, and from the nucleotide sequence, the primary amino acid sequence has been derived.[18, 19] Therefore it is now possible to investigate the T-cell epitopes in these proteins with the use of truncated recombinant proteins and synthetic peptides.

In this report to identify the T-cell epitopes present in *Der p* II, we synthesized overlapping peptides that cover the entire length of the molecule and screened them for their ability to induce proliferation of polyclonal T cells isolated from both HDM-allergic atopic individuals and normal healthy nonatopic control subjects. Using this approach, we demonstrate that both atopic and nonatopic individuals respond to *Der p* II. The proliferative responses of polyclonal T-cell populations indicate that, although T-cell epitopes are present throughout the protein, the sequences 11-50 and 61-104 contain the dominant

TABLE I. The polyclonal proliferative response of peripheral blood T cells to *Der p* II peptides

Subject	Medium	1-20	11-35	22-50	36-60	51-77	61-86	78-104	87-112	105-129	HDM
A1	1.6	5.6	13.9	12.7	7.0	3.8	54.6	35.7	17.5	7.0	61.2
A2	1.2	2.7	2.5	8.6	1.8	1.5	8.7	5.7	2.7	2.3	37.7
A3	1.0	2.2	3.2	3.5	13.4	2.0	10.0	5.9	5.5	22.9	25.5
A4	0.4	0.6	1.2	1.3	0.6	0.7	1.0	7.5	8.3	1.2	11.6
A5	3.0	5.0	6.8	4.3	4.5	4.2	8.1	3.8	4.1	4.7	33.5
A6	1.2	3.7	6.0	2.7	2.8	2.8	2.8	15.8	2.4	3.0	19.1
A7	0.9	0.9	1.5	1.6	0.9	1.5	5.9	1.7	1.5	2.2	72.7
A8	0.4	0.5	1.1	1.0	0.5	0.6	0.7	0.8	0.4	1.4	60.4
A9	1.3	2.0	2.7	3.0	2.5	3.4	2.8	3.7	2.4	1.3	36.9
NA1	2.1	4.4	4.8	9.5	2.5	2.4	22.0	14.6	2.8	5.3	71.9
NA2	0.3	0.6	0.6	0.5	0.8	4.5	4.0	3.1	0.5	0.8	6.1
NA3	1.0	4.0	3.5	8.0	10.5	10.9	9.3	13.3	3.2	3.8	13.9
NA4	0.3	0.3	0.4	0.6	0.5	2.6	3.3	1.1	0.4	4.3	8.3
NA5	1.2	1.6	3.5	2.1	4.0	2.0	2.8	5.6	2.6	1.7	56.2
NA6	0.4	0.5	0.6	0.5	0.5	1.0	1.2	1.0	1.1	0.8	18.4
NA7	0.7	0.7	1.0	0.7	0.7	0.7	0.7	0.7	0.8	1.0	11.1
NA8	0.6	0.7	0.7	0.7	0.8	0.7	0.8	1.5	0.6	0.7	31.0
NA9	1.4	1.4	3.1	1.4	1.4	1.4	3.5	2.1	1.4	1.7	11.9

PBMCs from atopic (A1 to A9) and nonatopic (NA1 to NA9) individuals were stimulated with the *Der p* II peptides over a concentration range of 0.1 to 50 μg/ml in 7-day cultures. The maximal response for each peptide is given as counts per minute $\times 10^{-3}$ and compared with the control response of PBMCs in medium alone. SEM was less than 25% in all experiments. Responses of greater than or equal to 2.5 times background are in boldface type.

T-cell epitopes. From the analysis of HDM-reactive T-cell clones a cluster of three distinct T-cell epitopes was identified within residues 11-35, and an additional two epitopes at residues 81-96 and 91-101.

METHODS

Antigens

Lyophilized unfractionated extracts of *D. pteronyssinus* were generously provided by Bencard (Brentford, Middlesex, England). Recombinant *Der p* II was cloned and expressed as previously described.[19] Peptides that covered the entire *Der p* II molecule (Fig. 1) were synthesized with solid-phase techniques on an Applied Biosystems (Foster City, Calif.) peptide synthesizer as described previously.[20] The purity of the peptides as determined by high-performance liquid chromatography was greater than 90%.

Characterization of patients

Atopic status with HDM allergy was defined by the presence of positive prick skin test results[21] (≥3 mm diameter wheal) to *D. pteronyssinus* and *D. farinae* extracts in the presence of a negative saline control. Nine HDM-allergic atopic subjects, aged 25 to 40 years, were included in the study. All of these individuals had clinical symptoms of HDM allergy (perennial rhinitis and/or asthma) and had grade III or IV serum-specific IgE for HDM as measured by RAST.

Nonatopic status was defined in a subject who was free of symptoms by the absence of positive skin prick test results to a panel of common environmental allergens (*D. pteronyssinus, D. farinae,* mixed grass pollen, birch pollen, *Aspergillus fumigatus, Cladosporium herbarum,* plane tree, nettle) in the presence of a positive wheal response to histamine hydrochloride (1 mg/ml) of ≥3 mm and a negative response to a saline control. Nine nonatopic individuals participated in the study. All had normal total IgE and negative specific IgE to the skin test panel of allergens as measured by RAST. No patients were receiving oral corticosteroid therapy or had previously undergone allergen-specific desensitization.

Isolation of human HDM-reactive clones

Cloned T cells reactive with *Der p* II peptides were isolated from HDM-allergic subjects by limiting dilution cloning from established T-cell lines as published elsewhere.[16, 22] Briefly, peripheral blood mononuclear cells (PBMCs; 10^6/ml) were stimulated with an optimal concentration of recombinant *Der p* II (10 μg/ml) for 7 days in RPMI-1640 medium supplemented with 2 mmol/L L-glutamine, 100 IU/ml penicillin/streptomycin (Gibco, Life Technologies, Paisley, Scotland) and 5% screened, inactivated human A+ serum (Blood Transfusion Service, Edgeware, England). Lymphoblasts enriched on Ficoll-Paque (Pharmacia Biotech Ltd., Milton Keynes, England) were established as a long-term line in the presence of irradiated autologous PBMCs (5×10^5/ml; 2500 rad), recombinant *Der p* II (20 μg/ml) and interleukin-2 (IL-2), (10% vol/vol; Lymphocult T,

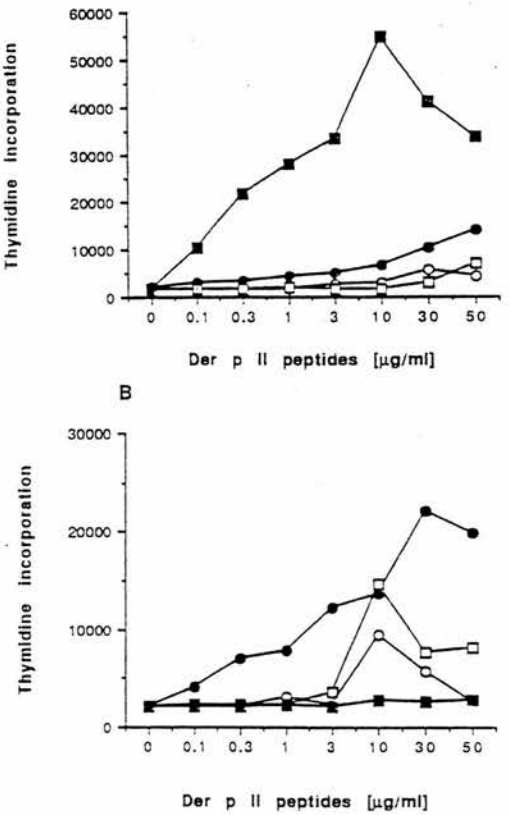

FIG. 2. The effect of peptide concentration on polyclonal T-cell proliferation to *Der p* II–derived peptides. Polyclonal T cells from an atopic individual were stimulated with different concentrations of the *Der p* II peptides 1-20 (○), 11-35 (●), 36-60 (□) and 61-86 (■) and proliferation (in counts per minute) was measured (**A**). In B the dose-dependent proliferation of T cells from a nonatopic individual to peptides 22-50 (○), 61-86 (●), 78-104 (□), and 87-112 (■) was measured.

Biotest Folex, Frankfurt, Germany) and subsequently cloned by limiting dilution. For cloning, viable cells (0.3 cells/well) were resuspended in supplemented medium and plated together with irradiated autologous PBMCs, *Der p* II, and IL-2. After 7 days, growing clones were expanded and maintained with IL-2 every 3 to 4 days, and irradiated autologous PBMCs and antigen were given every 7 days. Before use in proliferation assays, the clones were rested for 7 to 8 days after the last addition of antigen-presenting cells (APCs) and antigen.

Proliferation assays

Polyclonal T-cell responses. Unfractionated PBMCs (2.5×10^5/ml) were cultured with unfractionated *D. pteronyssinus* extract (20 μg/ml), recombinant *Der p* II (10 μg/ml), and *Der p* II peptides (0.1 to 50 μg/ml). After 6 days' incubation, the cultures were pulsed with tritiated methyl thymidine (1 μCi/well, Amersham International Inc., Amersham, England) and harvested 8 to 16 hours later. Proliferation as correlated with tritiated methyl thymidine incorporation was determined by liquid scintillation spectroscopy. The results were expressed as mean counts per minute for triplicate cultures with SEM less than 25% for all experiments with PBMCs.

Clonal T-cell responses. Cloned T cells (10^5 cells/ml) were stimulated with recombinant *Der p* II (10 μg/ml) or *Der p* II peptides (0.01 to 30 μg/ml) in the presence of an equal number of irradiated (2500 rad) autologous PBMCs as APCs. After 60 hours of incubation, tritiated methyl thymidine was added to the cultures for 8 to 16 hours, and they were then harvested as described for the polyclonal T-cell proliferation assays. The results are expressed as mean counts per minute for triplicate cultures with SEM less than 15% for all experiments with cloned T cells. All experiments were repeated with similar results on at least three separate occasions.

RESULTS

Response of polyclonal T cells to *Der p* II peptides

To identify those regions of *Der p* II in which the major epitopes recognized by T cells are located, proliferative responses of PBMCs to unfractionated HDM extract and overlapping peptides derived from *Der p* II were compared for nine HDM-allergic atopic (donors A1 to A9, Table I) and nine nonatopic (donors NA1 to NA9, Table I) individuals. T cells from both groups of subjects proliferated to HDM extract, with responses ranging from 6.1 to 72.7×10^3 cpm, and in general, the magnitude of the response of atopic subjects exceeded that of the nonatopic subjects. The background proliferation of unstimulated T cells cultured in medium alone was in the range of 0.3 to 3×10^3 cpm (Table I). Comparison of the maximal T-cell response to each *Der p* II peptide with the medium control, for the panel of individuals investigated here, revealed that each peptide induced proliferation in at least one donor. Furthermore, when we used the criterion that proliferation equal to or greater than 2.5 times the background response of cells cultured in medium alone constitutes a positive response (highlighted in boldface type in Table I), the majority of subjects responded to one or more of the peptides. An exception was donor NA7 who failed to show a proliferative response to any of the peptides, despite a proliferative response of 10.3×10^3 cpm to the intact recombinant *Der p* II

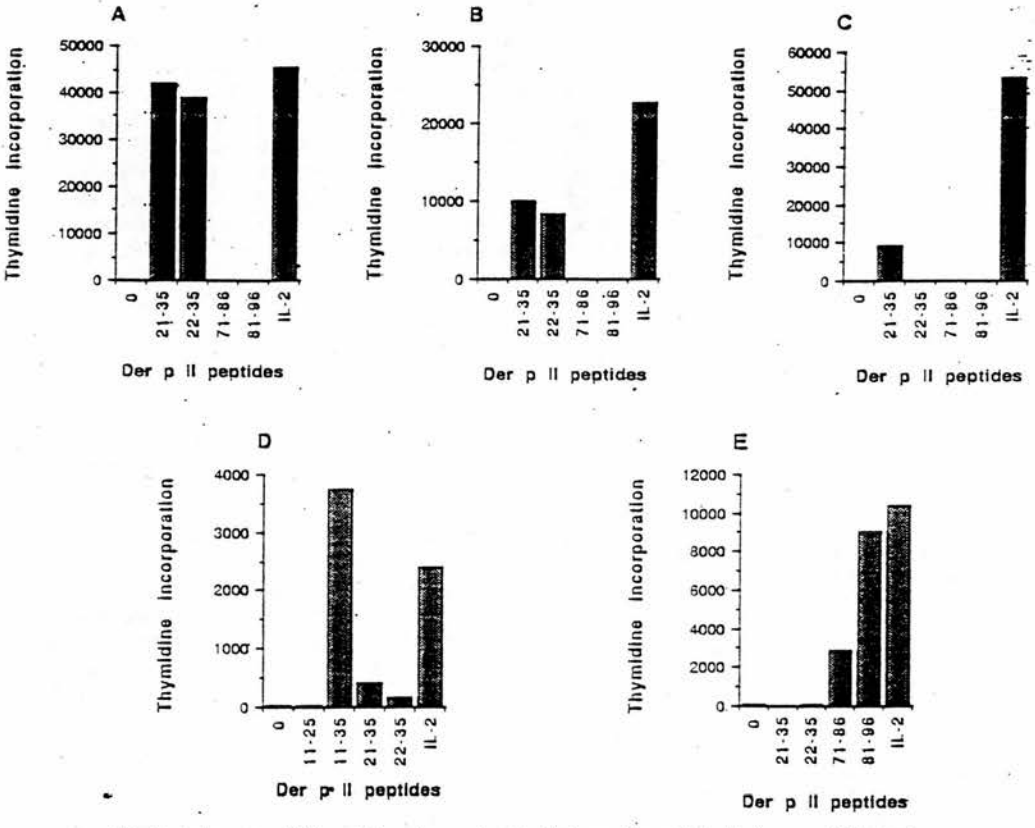

FIG. 3. Antigen specificity of *Der p* II–reactive T-cell clones. Cloned T cells from an HDM-atopic individual were stimulated with *Der p* II peptides in the presence of APCs, and proliferation was measured.

protein. The sequence 1-20 was the least antigenic of the peptides tested, in that for each of the subjects there were at least two peptides that stimulated greater proliferation; nevertheless, in donors A1, A6, and NA3 *Der p* II residues 1-20 induced stimulation indices of 3.5, 3.1, and 4, respectively (Table I). T-cell recognition of the *Der p* II peptides varied among individuals, and no pattern of proliferation that was unique to either the atopic or the nonatopic groups was observed (Table I). However, it appeared that both the atopic and nonatopic groups recognize predominantly T-cell determinants located within the region of 61-104 of *Der p* II, in that 16 of the 18 individuals had strong responses to peptides 61-86 and/or 78-104. Nine of the 18 subjects also responded to peptides spanning the region 11-50 (peptides 11-35 and 22-50). The sensitivity of the polyclonal T cells to different concentrations of peptide as illustrated in the dose-response curves of two individuals, one atopic subject (donor A1; Fig. 2, *A*) and one nonatopic subject (donor NA1; Fig. 2, *B*) may result from a number of different effects such as variation in T-cell receptor affinities (Fig. 2). The induction of T-cell nonresponsiveness may account for the reduction in proliferation observed at higher concentrations of peptide as observed in the response of donor A1 to peptide 61-86, for example (Fig. 2, *A*). The influence of peptide concentration on proliferation, as illustrated in Fig. 2, reinforces the need to assay peptides over a wide range of concentrations to determine whether or not the T cells of a given individual are reactive. The failure of any one of the *Der p* II peptides to induce proliferation in all individuals indicates that these peptides do not have the properties of antigen-nonspecific mitogens.

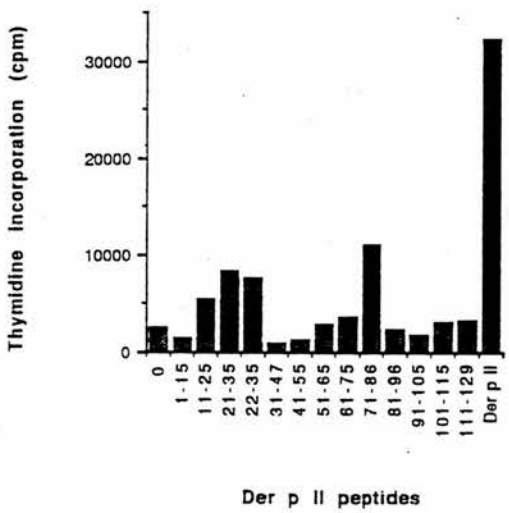

FIG. 4. Polyclonal T-cell response to *Der p* II peptides by HDM-allergic atopic individual from whom the T-cell clones shown in Fig. 3 were derived.

Isolation and characterization of *Der p* II reactive T-cell clones

In order to identify those regions of *Der p* II that constitute the major sites of T-cell recognition, PBMCs from an HDM-allergic atopic individual were stimulated with recombinant *Der p* II under optimal conditions and cloned by limiting dilution. Four of the *Der p* II–induced T-cell clones responded to epitopes at the amino terminal end of the molecule located between residues 11 and 35 (Fig. 3, *A* to *C*). Two of the T-cell clones proliferated to peptides 21-35 and 22-35 (Fig. 3, *A* and *B*), whereas one T-cell clone responded to only peptide 21-35 and not the sequence 22-35 (Fig. 3, *C*). The fourth T-cell clone was reactive with the N-terminus of *Der p* II, recognizing residues 11-35 but with considerably reduced proliferation to the peptide 21-35 (Fig. 3, *D*). An additional T-cell clone that responded strongly to the peptide containing residues 81-96 but less well to 71-86 was isolated. However, the ability of both peptides to induce proliferation of the T-cell clone suggests that the core residues of the epitope are associated with amino acids 81-86 (Fig. 3, *E*). In accordance with these results, the polyclonal T cells of this donor responded to recombinant *Der p* II and to the *Der p* II peptides 11-25 and 21-35, with the dominant response directed to residues 71-86 (Fig. 4). In contrast, proliferation to residues 81-96 was more limited (Fig. 4).

From the peripheral blood of a second HDM-allergic atopic individual, T-cell clones were induced with recombinant *Der p* II. Analysis of the antigen specificity of the cloned T cells isolated from this atopic subject revealed that they respond to peptide 91-105 but not to residues 81-96 or 101-115, suggesting that the epitope maps to residues 91-101 (Fig. 5). At the polyclonal level the T cells from this donor proliferated strongly to peptides containing residues 51-77, 61-86, and 78-104 (Fig. 6). Thus the presence of major T-cell determinants suggested by analysis of the polyclonal response are reflected at the clonal level.

DISCUSSION

Although initially the major allergenic determinants were thought to reside in the group I allergens of HDM, recent analyses of specific antibody[8-12] and T-cell responses[16, 17] indicate that immune recognition of the group II allergens is an equally important component of the allergic response to HDM. In this study, designed to investigate the specificity of human T-cell responses to the group II allergen of *D. pteronyssinus* at the epitope level, we demonstrate, using overlapping peptides, the presence of multiple T-cell epitopes located in all regions of the protein capable of stimulating T cells from both atopic and nonatopic individuals. Furthermore, we observed that the majority of both atopic and nonatopic individuals examined here respond to T-cell epitopes located within regions 11-50 and 61-104.

The analysis of the proliferative response of polyclonal T-cell populations to intact *Der p* II expressed as a recombinant protein and synthetic peptides indicated that components of the T-cell repertoire of both atopic and nonatopic individuals are reactive with *Der p* II, although quantitatively the responses may differ. One nonatopic donor, however, although able to recognize the full-length *Der p* II molecule, failed to respond to any of the peptides tested. Differences in the amino acid sequences of "naturally processed" T-cell epitopes as compared with the peptides that were synthesized may account for this observation. The findings reported here support our previous observations that HDM-reactive T cells are present in the peripheral T-cell pool of both atopic and nonatopic individuals.[16] In contrast, it has been reported that peripheral T cells from nonatopic individuals fail to proliferate to *Der p* II,[17] as well as *Der p* I.[15, 17] Furthermore, in the studies described here, it appears that the polyclonal T cells from both groups of subjects proliferate more vigorously to sites located between 61

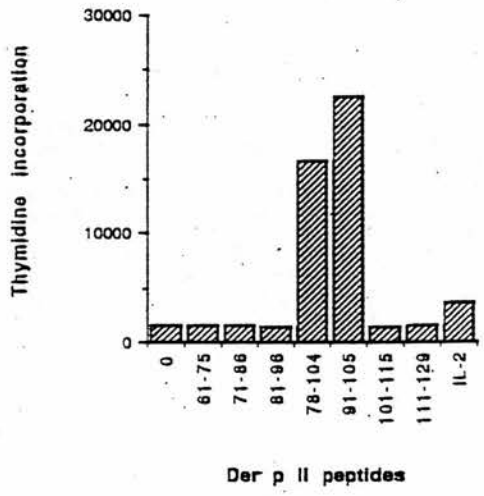

FIG. 5. Antigen specificity of *Der p* II–reactive T-cell clones. Cloned T cells from a HDM-atopic individual were stimulated with *Der p* II peptides in the presence of APCs.

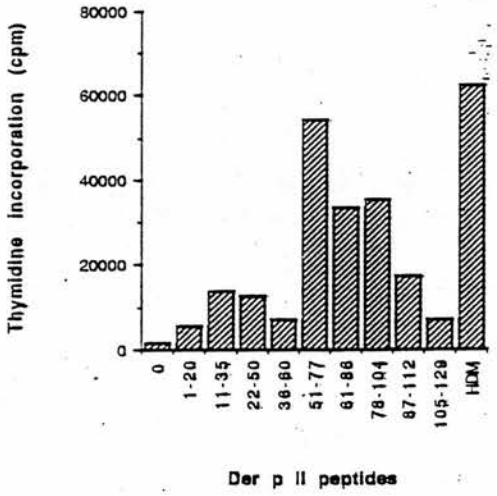

FIG. 6. Polyclonal T-cell response to *Der p* II peptides by HDM-allergic atopic individual from whom the T-cell clones shown in Fig. 5 were derived.

and 104 than to other sequences in *Der p* II. The reason that this region of the protein is immunodominant has not been defined but may reflect the presence of peptides capable of binding with high affinity to a wide range of major histocompatibility complex class II molecules. The majority of individuals responded to two or more peptides, and multiple T-cell epitopes appear to be distributed throughout the protein, as has been observed for *Der p* I,[23] with no apparent immunologically silent regions. Furthermore, no characteristic patterns of proliferation that allowed atopic and nonatopic subjects to be differentiated were observed, which may reflect differences in the HLA haplotype of the different individuals studied.

The presence of T-cell epitopes in *Der p* II was examined further by analyzing the antigen specificity of *Der p* II–induced T-cell clones isolated from the peripheral blood of atopic individuals. In the first of the atopic individuals (whose polyclonal T cells responded to residues 11-25, 21-35, and 71-86), four T-cell clones that recognize determinants at the amino terminus of the molecule were isolated. From the analysis of the fine antigen specificity of these T-cell clones, three closely related epitopes within residues 11-35 were identified. Another T-cell clone that was isolated from the same individual responded to stimulation with residues 81-96, even though the polyclonal T-cell response to this peptide was weak. With overlapping peptides from this region of *Der p* II, it was observed that peptide 71-86, which was immunodominant at the polyclonal level, did not contain the optimal T-cell epitope recognized by the cloned T cells. For another atopic donor, from whom *Der p* II–specific T cells were cloned, their specificity for residues 97-105 overlapped with one of the regions identified at the polyclonal level. However, the most marked proliferative response of the unfractionated T cells was to residues 51-77. In both donors the antigen specificity of the T-cell clones and the polyclonal response overlapped, indicating that T-cell clones may provide a valid reflection of the T-cell repertoire as a whole. When synthetic peptides were used to probe the specificity of *Der p* II–reactive IgE, a B-cell epitope in the same region (residues 65-78) of the molecule was mapped.[24] Although T cells and B cells tend to recognize distinct epitopes within a protein,[25] there are instances in which it has been demonstrated that they overlap.[26] However, the majority of the *Der p* II peptides failed to bind IgE, which may reflect an alteration in the conformation of B-cell sites as compared with the intact protein.[27]

The identification of the major T-cell determinants in allergens has practical applications in the design of immunotherapeutic agents for clinical desensitization, and considerable interest has been directed to the use of peptides for redirecting[28] or downregulating allergic immune responses.[29] Although we observed that the region

encompassed by residues 61-104 (peptides 61-86 and 78-104) is recognized by 89% of the individuals tested in this study, the overall heterogeneity of the antigen specificity of the *Der p* II–reactive T-cell repertoire suggests that peptide-based hyposensitization may be difficult to achieve. This will be further complicated by the differential sensitivity of polyclonal T cells to different concentrations of particular peptides. Furthermore, the region of the protein encompassing residues 61-104 contains an epitope that binds *Der p* II–specific IgE,[24] and therefore therapy based on peptides that contain the dominant T-cell epitopes in this instance may induce adverse reactions in patients with serum antibodies of this specificity. Even if nonresponsiveness were induced to dominant epitopes, it is possible that other minor regions may trigger allergic immune responses.[30] In the murine model of experimental allergic encephalomyelitis, during the induction phase of the disease, the T-cell response is directed toward an immunodominant site at the amino terminus of myelin basic protein.[31] However, as the disease becomes chronic, or if encephalomyelitis is induced with the amino terminal peptide alone, additional determinants that are cryptic after the initial immunization then become immunogenic.[32] This suggests that desensitization with polypeptide fragments of *Der p* II, in which the B-cell sites have been silenced, may offer an alternative approach to inducing nonresponsiveness to *Der p* II.

We thank Dr. M-C. Kuo of the ImmuLogic Pharmaceutical Corporation in Waltham, Mass., for providing some of the peptides used in this study.

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International Immunology, Vol. 8, No. 7, pp. 1091–1098

Induction of T cell responses to the invariant chain derived peptide CLIP in mice immunized with the group 1 allergen of house dust mite

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Keywords: CLIP, house dust mite allergen, invariant chain, murine T cell, T cell cross-reactivity

Abstract

In this study we demonstrate that immunization of H-2^b mice with the allergen Der p 1 induces MHC class II restricted T cells that proliferate to residues 15–29 of Der p 1 (p15–29) and to the murine MHC class II-associated invariant chain derived peptide (CLIP). T cells from naive H-2^b mice and those immunized with murine CLIP fail to respond to either CLIP or p15–29. T cell lines and clones reactive with p15–29 strongly proliferate in response to splenic antigen-presenting cells (APC) from normal H-2^b mice but show reduced proliferation to APC from invariant chain deficient mice. Furthermore, T cells isolated from Der p 1 primed mice and expanded on H-2^b spleen cells in the absence of the p15–29 epitope retained specificity for both p15–29 and CLIP, suggesting that naturally presented self components can act as mimetic peptides and may maintain T cell memory to foreign antigens.

Introduction

The group 1 allergen of house dust mite (*Dermatophagoides pteronyssinus*, HDM) is a major target of the IgE response in HDM atopic individuals (1) and allergen-specific CD4$^+$ T cells play a central role in the regulation of allergic inflammatory responses (e.g. 2,3). From several experimental models there is evidence to suggest that it is possible, using allergens or their derivatives, to modulate the function of specific CD4$^+$ T cells (e.g. 4–6). Although a significant proportion of naturally processed peptides bound to MHC class II molecules are derived from self antigens (7–10), the peripheral T cell repertoire remains unresponsive to self. Such observations have generated interest in the potential of certain peptides derived from self molecules, such as MHC class II, to inhibit T cell recognition of foreign antigens (e.g. 11). This prompted us to investigate the efficacy of a non-immunogenic I-A^b binding peptide, i.e. the invariant (Ii) chain derived CLIP peptide, in down-regulating murine immune responses to HDM allergens, as an alternative approach to using derivatives of HDM.

Newly synthesized MHC class II α and β chains associate in the endoplasmic reticulum (ER) and form complexes with the Ii chain. The Ii chain molecule contains signal sequences that guide nascent MHC class II to the appropriate endosomal compartment (12,13) and prevents the binding of endogenous peptides in the ER (14–16). The Ii chain is removed from the MHC class II molecule by stepwise proteolysis to allow the acquisition of peptides. This results in the generation of a series of nested CLIP peptides, which are encoded by the third exon of the Ii chain gene (17). CLIP peptides have been eluted from a subset of murine and human class II MHC molecules, and appear to bind to a marked proportion of the intracellular pool of MHC class II molecules (7–10,18). CLIP can bind to MHC class II molecules *in vitro* with similar binding kinetics to traditional antigenic peptides (19). Furthermore, the recent resolution of the crystal structure of CLIP–HLA-DR3 complexes (20) shows that CLIP occupies the antigen binding groove in a manner almost identical to that of a peptide antigen. Relatively high levels of CLIP–MHC class II complexes can be detected on the surface of human B

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Transmitting editor: D. Mathis

Received 4 January 1996, *accepted* 28 March 1996

lymphoblastoid cell lines (21). However, it remains to be established whether such complexes reach the cell surface of normal cells. If they do, it appears that they fail to induce T cell responses in the periphery either because they are not present in sufficient density or the peripheral T cell repertoire is tolerant to them. Consequently, immunizing mice with murine CLIP has failed to induce T cell responses (22), whereas specific T cells can be primed in mice with human CLIP (23).

Mimicry between self and foreign antigens has been mooted as a potential mechanism for breaking self tolerance leading to the development of autoimmunity (e.g. 24,25). The observation that peptide analogues, derived from common human pathogens, can act as molecular mimics for autoreactive human $CD4^+$ T cell clones specific for myelin basic protein (MBP) supports this hypothesis (26). However, the converse may also hold true, i.e. that peptides derived from self antigens may have the capacity to activate memory T cells already primed to foreign antigens. By the variation in sequence these self derived peptides may function as partial agonists or altered T cell ligands. It appears that variants of self peptides may arise naturally *in vivo* and thus have the potential to influence the selection of the repertoire (e.g. 27,28). Changes in the affinity of TCR–ligand interactions can modify the effector function of mature T cells, and the state of T cell differentiation may also influence both quantitative and qualitative aspects of signals delivered by altered T cell ligands (29–34).

Here we report that a peptide located in the N-terminus of Der p 1, p15–29, shares a high degree of sequence homology to the CLIP peptide. $CD4^+$ T cells primed *in vivo* with Der p 1 respond to the HDM peptide but also to murine CLIP. T cell responses to CLIP or p15–29 could not be induced by immunizing with the murine CLIP peptide or with an irrelevant foreign antigen. The cross-reactivity was further investigated using oligoclonal T cell lines, T cell clones and hybridomas, isolated from Der p 1 primed mice, and we demonstrate that p15–29-reactive $CD4^+$ T cells can be induced to proliferate, in the absence of exogenous peptide, by the addition of increasing numbers of spleen cells from normal H-2^b mice. We propose that CLIP acts as a weak agonist for the Der p 1-specific T cells through molecular mimicry rather than acting as an altered T cell ligand. The ability of self peptides to perpetuate responses to allergen-derived peptides is relevant, not only in the pathogenesis of allergic inflammation, but also in the development of immunotherapy.

Methods

Mice and cell lines

C57BL/6J female mice were purchased from Harlan (Chester) UK at 6–8 weeks of age and maintained under conventional conditions. C57BL/6J mice homozygous or heterozygous for a disruption of the Invariant chain gene were a kind gift from Drs D. Mathis and C. Benoist (IGBMC, Strasbourg, France), and have been described elsewhere (35). The BW 5147 ($TCR\alpha\beta^{-/-}$) fusion partner was a kind gift from Dr H. Bodmer (Nuffield Department of Clinical Medicine, Oxford, UK). The ovalbumin (OVA) 323–339 specific T cell hybridoma BO 17.10 was a kind gift from Dr C. Hauser (Department of Dermatology, Hospital Cantonal Universitaire, Switzerland).

Immunization

Mice were primed s.c. at the base of the tail with 50 μg of antigen in complete Freund's adjuvant (CFA; Sigma, St Louis, MO). Periaortic and inguinal lymph nodes were collected 8–10 days post immunization, teased with frosted glass slides and washed. The single cell suspension was used in proliferation assays or for long-term culture.

Antigens and antibodies

Der p 1 protein was affinity purified from spent mite medium as described previously (36). Peptides were synthesized by standard solid phase methods, on an ABI 431 peptide synthesizer (Foster City, CA), using F-moc chemistry. Peptides were based on the sequences of: Der p 1 (p15–29, p113–127 and p111–139), the murine Ii (murine CLIP residues 82–104), the human Ii (human CLIP residues 99–122) and the I-A^b-restricted OVA peptide 323–339 (37–40).

Culture medium

Cells were cultured in RPMI 1640 supplemented with 5% FCS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin (Gibco, Grand Island, NY) and 50 mM 2-mercaptoethanol (Sigma, St Louis, MO).

Isolation of long-term T cell lines, T cell clones and hybridomas

Lymph node cells from Der p 1 primed mice were cultured at 3×10^6 cells/ml with 10 μg/ml Der p 1 (line F2.2) or p111–139 (line F1) in 1.5 ml final volume in 24-well plates (Nunc, Roskilde, Denmark). T cells were then re-stimulated several times in 25 cm^2 vented culture flasks (Costar, Cambridge, MA) every 10–14 days with 5 μg/ml p111–139 (F1) or 10 μg/ml Der p 1 (F2.2) at a 1:30 ratio of T cells to antigen-presenting cells (APC; C57BL/6J spleen cells irradiated with a dose of 2500 rad).

Resting T cells from a Der p 1-specific long-term T cell line were cloned by limiting dilution with the peptide p15–29, 5×10^5 APC/well and 20 ng/ml human recombinant IL-2 (Biogen, Boston, MA). Selected clones were subcloned by limiting dilution on either p15–29 or murine CLIP as above. Der p 1-specific T cell hybridomas were generated by polyethylene glycol (Sigma)-induced fusion of Der p 1-specific T cell clones and lines with the BW 5147 ($TCR\alpha/\beta^{-/-}$) fusion partner and selecting with HAT medium (Sigma).

Proliferation and cytokine assays

Lymph node cells from primed mice were cultured at 2.5×10^5 cells/well for 72 h. T cell lines or clones were cultured at $2–3\times10^4$ cells/well in the presence of $8–10\times10^4$ APC [irradiated 2500 rad or mitomycin C (Sigma)-treated C57BL/6J spleen cells] for 48 h. Cells were cultured in triplicates in 200 μl medium/well in round-bottom plates (Nunc, Roskilde, Denmark), either alone or in the presence of varying doses of antigen and were pulsed for the last 6 h with 0.5 μCi [^{3}H]thymidine/well (Amersham Life Sciences). Cells were harvested onto glass fibre filters and proliferation was determined as the incorporation of [^{3}H]thymidine, measured by a liquid scintillation counter 1205 Betaplate (Wallac).

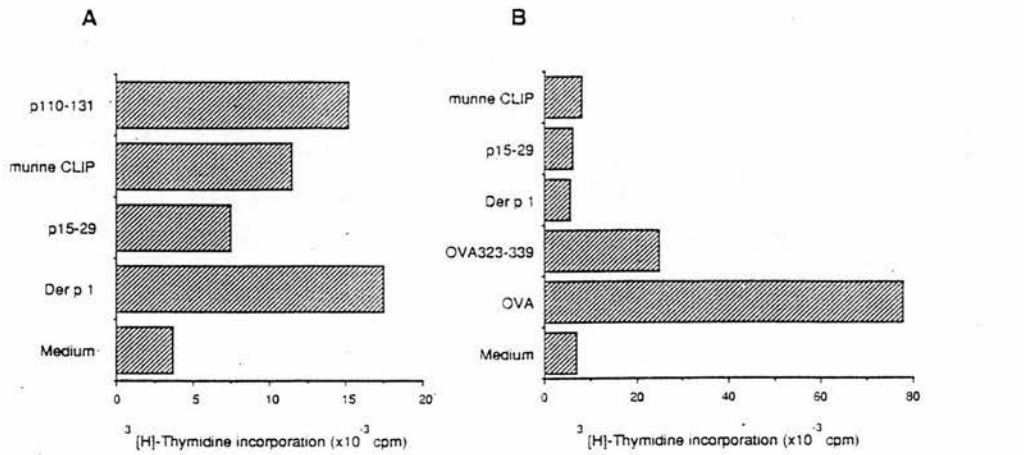

Fig. 1. T cells from mice immunized with Der p 1, but not with OVA, respond to murine CLIP peptide *in vitro*. Lymph node cells from mice primed with either (A) Der p 1 or (B) OVA were tested for IL-2 production and proliferation in response to the indicated antigens. Similar response profiles are seen with IL-2 production and proliferation. The responses shown are geometric means of IL-2 responses to 5 μM peptide, 15 μg/ml Der p 1 and 800 μg/ml OVA.

To measure IL-2 production T cells were cultured as above and after 24 h incubation 50 μl culture supernatant was collected. Activation of T cell hybridomas was measured by culturing 5×10^4 hybridoma cells in 200 μl of media with APC and peptide for 20 h and assaying the IL-2 contents of the supernatants.

IL-2 was measured in culture supernatants using the IL-2-dependent cell line CTLL-2. Briefly, 5000 cells in 50 μl medium were cultured in the presence of 50 μl supernatant for 24 h. Cells were pulsed for 6 h with 0.5 μCi [^{3}H]thymidine/well and proliferation was determined as above.

Results

Immunization with Der p 1 induces polyclonal T cell responses to an amino terminal Der p 1 epitope (residues 15–29) and to murine CLIP

Prior to investigating the efficacy of CLIP in the modulation of HDM-specific T cell responses, we assessed the ability of this peptide to induce polyclonal T cell responses in H-2^o mice primed with Der p 1. Regional lymph node cells from mice immunized with Der p 1 were tested *in vitro* with increasing concentrations of HDM-derived antigens or murine CLIP. T cells respond in a dose-dependent manner to intact Der p 1, a peptide containing the immunodominant CD4-restricted Der p 1 epitope (p110–131) and to murine CLIP (Fig. 1A). The CLIP peptide is not mitogenic since it fails to stimulate proliferative responses or IL-2 production in T cells from naive H-2^o mice (data not shown) or mice immunized with OVA, an unrelated antigen (Fig. 1B). Although Der p 1-induced T cell responses can be recalled by stimulation with the CLIP peptide *in vitro*, immunization with either murine or human CLIP itself in CFA has failed to prime T cell responses to either CLIP, Der p 1 or Der p 1-derived peptides (data not shown).

Comparison of the primary amino acid sequences revealed a high degree of homology between CLIP and the N-terminal region of Der p 1 (Table 1). MHC class II binding motifs have been predicted based on analysis of the sequences of known I-A^o-restricted peptides (Table 1). The homologous regions of CLIP and Der p 1 complies with one predicted motif (41), where a bulky amino acid (Phe, Tyr or Met) constitutes one anchor residue, separated by two to four variable residues from the second anchor residue, which is a non-polar amino acid residue (either Pro or Ala). Furthermore, it has been proposed that methionine residues in human CLIP may provide important MHC contact residues, facilitating binding to many different MHC class II molecules (42). This suggests that murine CLIP and the homologous Der p 1 peptide can be presented to T cells in an I-A^o-restricted manner. In order to assess whether or not this region of Der p 1 can be recognized by T cells from Der p 1-primed mice, a 15 amino acid peptide representing residues 15–29 of Der p 1 (p15–29) was synthesized. The p15–29 peptide has been tested over a wide concentration range and elicits strong *in vitro* proliferative responses as well as IL-2 production in lymph node cells from Der p 1-primed H-2^o mice (Fig. 1A), but not from mice primed with OVA (Fig. 1B).

T cell reactivity to p15–29 and murine CLIP is retained during long-term in vitro culture

In order to investigate whether or not specificity for p15–29 and CLIP was retained during long-term *in vitro* culture, the reactivity of Der p 1-specific T cell lines and clones was examined. T cell lines derived from Der p 1-primed mice proliferated in response to p15–29 and CLIP peptide, but

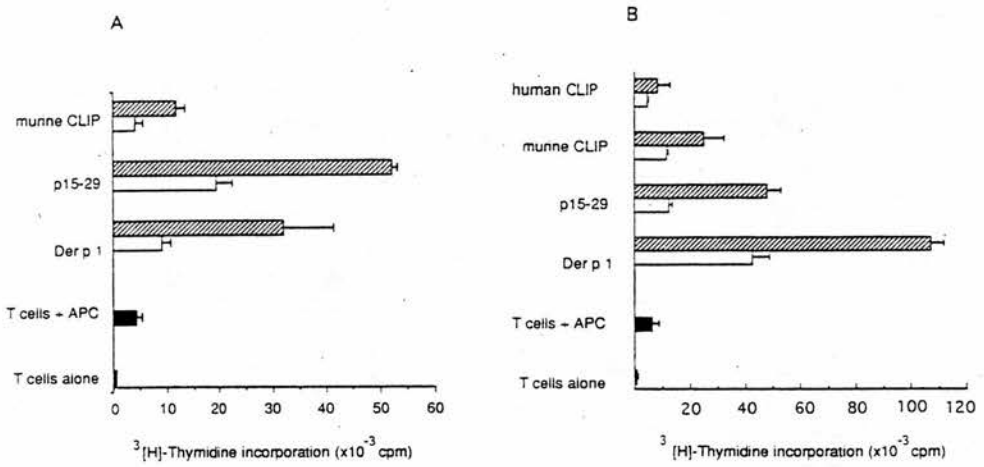

Fig. 2. Long-term T cell lines specific for HDM-derived peptides cross-react with murine, but not human, CLIP peptide. T cell lines were derived by culturing lymph node cells from mice primed with (A) Der p 1 (line F2.2) or (B) the peptide p111–139 (line F1) and re-stimulating several times with irradiated spleen cells and the relevant antigen. The data shown is proliferative responses of resting T cells in the absence (dark bars) or presence of 1 μg/ml (open bars) and 10 μg/ml (cross-hatched bars) of the indicated antigen.

Table 1. Comparison of primary amino acid sequences of HDM derived peptides and murine and human CLIP

I-Ab binding motif	N X X X X X P D I Q S	Rudensky et al. (18)
	F X X X X X P Y A M	Hobohm and Meyerhans (41)
Der p 1 (113-127)	I S N **Y** C Q I Y **P** P N A N K I	
Der p 1 (15-29)	D L R Q **M** R T V T **P** I R M Q G	
Murine Invariant chain (82-104)	P K S A K P V S Q **M** R M A T **P** L L M R P M S M	
Human Invariant chain (98-122)	P K P P K P V S K **M** R M A T **P** L L M Q A L P M G A	

The p113–127 peptide is the minimal CD4-restricted Der p 1 epitope (N. M. Kristensen, unpublished data). The peptides p110–131 and p111–139, which also contain this epitope and induce similar responses, have also been used in this study. Bold letters indicate potential MHC class II anchor residues according to the I-A^b binding motif suggested by Hobohm and Meyerhans (41). The I-A^b binding motif suggested by Rudensky *et al.* (18) is also shown.

more strongly to p15–29 than to murine CLIP, as illustrated by the pattern of responses of the T cell lines F2.2 and F1 (Fig. 2A and B). We furthermore isolated a panel of T cell clones from a long-term Der p 1-specific T cell line and selected T cell clones were subsequently subcloned on p15–29 or murine CLIP. All T cell subclones, regardless of the selecting peptide, responded more strongly to p15–29 than to murine CLIP, as illustrated by two representative subclones (Fig. 3A and B). The examined clones all produced IFN-γ as well as IL-2 upon antigen stimulation and the cytokine response profiles of each clone were qualitatively similar after stimulation with either p15–29 or murine CLIP (data not shown). In order to rule out the possibility that the p15–29-specific cells can be stimulated by any stable I-A^b–peptide complex, the responses of the clones to human CLIP were tested. This peptide shares a large degree of homology with murine CLIP and it has recently been shown to induce I-A^b-restricted responses (23). However, it failed to induce cytokine

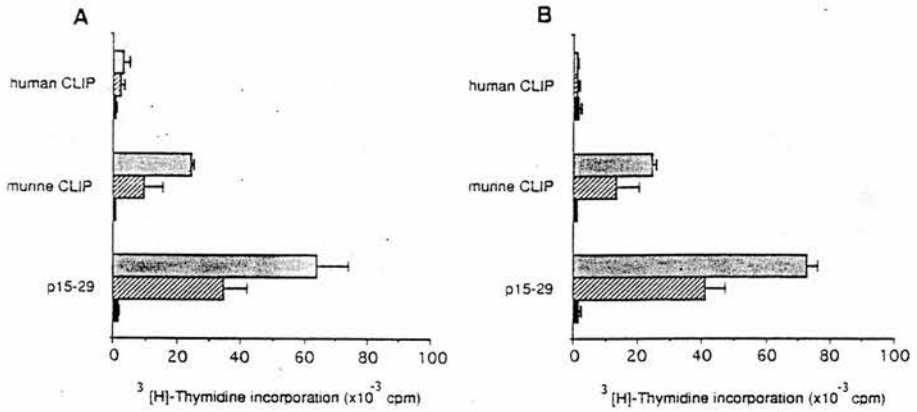

Fig. 3. T cell clones proliferate both to p15–29 and murine CLIP, but not to the I-A^{b} binding human CLIP. The responses shown are those of two representative clones to 0.5 μM (open bars) or 5 μM (cross-hatched bars) of the indicated peptides. Background proliferation (± SD) in the presence of APC was (A) 1135 ± 589 c.p.m. and (B) 1857 ± 1357 c.p.m.

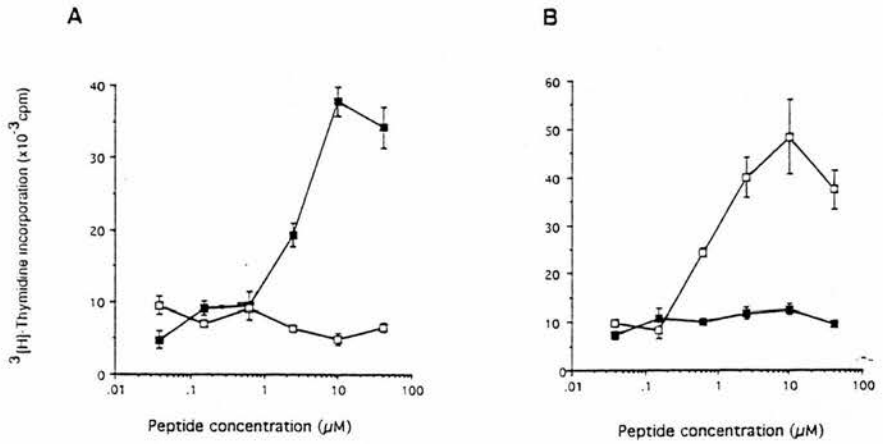

Fig. 4. T cell hybridomas specific for p15–29 cannot be activated by I-A^{b} complexed with the unrelated I-A^{b}-restricted OVA peptide, whereas an OVA-specific T cell hybridoma can. T cell hybridomas were assayed for IL-2 production after culture with increasing doses of p15–29 (filled squares) or OVA323–339 (open squares) and APC. The results presented are for a representative p15–29-specific hybridoma (A) and the OVA323–339-specific hybridoma BO 17.10 (B). Background proliferation (i.e. T cell hybridomas cultured with APC only) was (A) 6189 ± 657 c.p.m. and (B) 8447 ± 516 c.p.m.

production or proliferation (Fig. 3 and data not shown). Furthermore, a set of T cell hybridomas was generated and tested against a wide concentration range of the I-A^{b}-restricted epitope OVA323–339. None of the p15–29/CLIP-specific hybridomas responded to the OVA epitope, as illustrated by a representative hybridoma (Fig. 4A).

The line F1 was isolated from H-2^{b} mice immunized with Der p 1, but selected *in vitro* with splenic APC and the peptide p111–139, which contains the dominant I-A^{b}-restricted Der p 1 epitope p113–127 (N. Kristensen, unpublished data). Although F1 thus was selected in the absence of the p15–29 epitope it proliferated strongly to p15–29 and murine CLIP as well as to Der p 1 (Fig. 2B). The outgrowth of p15–29/murine CLIP-specific T cells in this line may be attributed to the combination of bystander help provided by T cells stimulated to release cytokines by the immunodominant epitope and the high number of APC, supplying a sufficient density of CLIP–MHC class II complexes. We observed markedly higher levels of proliferation for T cell lines and clones cultured in the presence of APC than in medium alone (e.g. Fig. 2A and B). These findings suggest that the T cell reactivity to CLIP and p15–29 may be maintained by splenic APC in the absence of exogenous antigen. Therefore, we investigated the proliferative responses to increasing doses of splenic APC

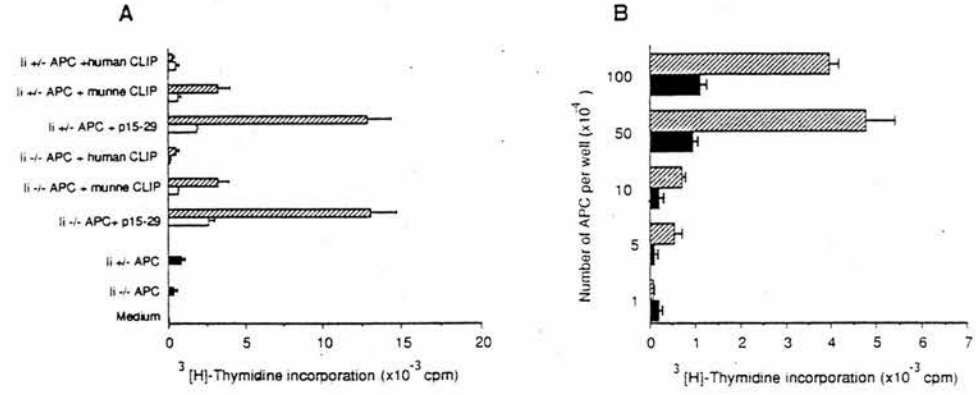

Fig. 5. Antigen-presenting capacity of spleen cells from Ii chain deficient mice and control heterozygous littermates. (A) T cells from the line F2.2 were cultured with 0.5 μM (open bars) or 5 μM (cross hatched bars) of the indicated peptides and mitomycin-treated spleen cells from C57BL/6J mice either heterozygous ($Ii^{+/-}$ APC) or homozygous ($Ii^{-/-}$ APC) for a mutation in the Ii chain gene. Proliferation to medium alone or spleen cells in the absence of peptide antigen (shaded bars as indicated) is also shown. (B) T cells were cultured with increasing numbers APC from of $Ii^{-/-}$ mice (cross hatched bars) or APC from $Ii^{-/-}$ (shaded bars).

from mice expressing Ii chain or deficient in Ii chain expression.

Proliferative responses of p15–29/murine CLIP-reactive T cells induced by splenic APC from normal and Ii deficient mice in the presence and absence of exogenous antigen

The capacity of APC from Ii chain deficient mice ($Ii^{-/-}$) and control heterozygous littermates ($Ii^{+/-}$) to present peptides *in vivo* was examined. Consistent with previous reports we found that APC from the $Ii^{-/-}$ mice, though expressing considerably reduced levels of membrane bound MHC class II molecules (35), could present the peptides p15–29 and murine CLIP as efficiently as APC from $Ii^{+/-}$ mice. No responses to human CLIP were observed (Fig. 5A). T cells from long-term T cell lines and clones were cultured with increasing numbers of APC from either $Ii^{+/-}$ or $Ii^{-/-}$ H-2^b mice in the absence of exogenous antigen. A dose-dependent increase in T cell proliferation was observed with APC from $Ii^{+/-}$ mice. However, the response of the T cells to $Ii^{-/-}$ APC was greatly reduced compared to that obtained with equivalent numbers of APC from $Ii^{+/-}$ mice (Fig. 5B).

Discussion

In this report, we demonstrate that $CD4^+$ T cells specific for a peptide (residues 15–29) derived from a major allergen of HDM, Der p 1, are cross-reactive with an Ii chain processing intermediate (CLIP, residues 82–104). The observation that immunization with an allergenic protein can stimulate T cell responses to murine CLIP raises the possibility that self peptides may play a role in the maintenance of immunological memory of T cells specific for foreign antigens by generating cross-reactive responses *in vivo*.

From comparison of the primary amino acid sequences a region located within the N-terminus of Der p 1 was identified with marked similiarity to murine CLIP (Table 1) and it is likely that the homology between the two sequences accounts for the T cell cross-reactivity. The resolution of the crystal structure of the CLIP–HLA-DR3 complex (20) and the reports that CLIP binding to MHC class II molecules has similar kinetics to conventional antigenic peptides, and is influenced by allelic polymorphism (19,42,43), suggest that CLIP has the binding properties of a conventional peptide. Furthermore, I-A^b-restricted responses to the highly homologous human CLIP have recently been reported (23). The responses seen with murine CLIP did not occur as the result of mitogenic or superantigen effects, since we failed to detect CLIP-reactive T cells in mice immunized with an irrelevant antigen (OVA) and CLIP-reactive T cells could not be isolated from OVA-reactive T cell lines.

It could be argued that the Der p 1-specific T cells are cross-reactive with an epitope on I-A^b which is exposed upon stable peptide binding, and that the T cells therefore can be stimulated by any I-A^b–peptide complex. However, neither human CLIP nor an I-A^b-restricted OVA peptide could induce responses in p15–29-specific T cell lines, clones and hybridomas. Thus, it appears that p15–29-specific T cells induced by immunization with Der p 1 can specifically recognize murine CLIP when presented as exogenous peptide *in vitro*.

It still remains to be resolved if CLIP–MHC class II is constitutively expressed on the surface of normal APC. Nevertheless, p15–29-specific T cells could be induced to proliferate *in vitro*, in the absence of exogenous peptide by the addition of splenic APC from normal H-2^b mice. If CLIP is the ligand in normal H-2^b APC inducing proliferation it might be expected that splenic APC from $Ii^{-/-}$ mice would be unable to elicit a response. The proliferation was greatly reduced with APC from $Ii^{-/-}$ mice; however, it was not completely abrogated. Adding murine CLIP to the $Ii^{-/-}$ APC restored strong T cell responses. There are several explanations to account for the residual proliferation in the presence of $Ii^{-/-}$ APC, including

bystander help mediated by the release of cytokines from APC, when added at high density. Additionally, it cannot be excluded that the T cells we describe here can respond to more than one self peptide and it is recognition of unidentified peptides that induces proliferation seen with H-2^0 APC in the absence of exogenous peptide. Nevertheless, the finding that CLIP can induce proliferation of T cell clones and lines specific for p15–29, suggests that CLIP is a cross-reactive ligand recognized by these T cells. Moreover, we have subcloned the cross-reactive T cells using murine CLIP and the T cells isolated maintain stronger responses to the HDM peptide than to CLIP. These results would support CLIP as a ligand for the HDM-reactive T cells.

Cross-reactivity between self and foreign antigens occurring *in vivo* may have the potential to break peripheral tolerance and lead to the induction of autoimmune disease (e.g. 24,25). In order to activate self reactive CD4$^+$ T cells foreign peptides must be able both to bind to MHC class II molecules and form productive interactions with the TCR. Recently, it has been described that autoreactive human CD4$^+$ T cell clones specific for MBP could be stimulated *in vitro* by peptides derived from common human pathogens (26). The mimetic peptides displayed only limited sequence homology to the MBP peptide, nevertheless, they all stimulated strong proliferative responses in the appropriate T cell clones. If foreign antigenic peptides can activate self reactive T cells through molecular mimicry, then the converse may also be true in that T cells specific for a foreign antigen may be stimulated by mimetic self peptides. Thus T cells primed to selected foreign antigens, such as Der p 1, may be maintained *in vivo* by recognition of naturally occurring self peptides, which act as weak agonists or altered T cell ligands, when presented on the surface of professional APC. While CLIP may be unable to activate naive p15–29/CLIP-specific T cells, memory T cells which have previously encountered p15–29 may have a lower activation threshold, which enables CLIP to act as an agonist. Signalling through ligation of the TCR by peptide–MHC class II complexes with altered affinities can dissociate selective T cell effector functions (44,45). Our results indicate that recall responses to murine CLIP are consistently lower than those to the original antigen p15–29, however, both ligands induce proliferation and similar profiles of cytokine production. Thus, murine CLIP acts as a weak agonist rather than an altered T cell ligand.

The failure of the human CLIP peptide to stimulate the p15–29-reactive T cells is interesting since murine and human CLIP are more homologous to each other than to the HDM peptide. A recent report has proposed that the minimal epitope necessary for binding of human CLIP to I-A^b comprises amino acid residues 117–125, which are identical to the corresponding region in murine CLIP (residues 91–99) (23). Assuming that p15–29 binds to I-A^0 in a manner similar to human CLIP, comparison of the amino acid sequences of p15–29, murine and the non-stimulatory human CLIP suggests that the primary TCR contact residue could be Gln at position 18 in p15–29, corresponding to position 90 in murine CLIP. At the corresponding position in human CLIP there is a non-conservative substitution of Gln to Lys at position 106 and this change would be sufficient to abrogate T cell recognition if the residue at this position is a primary TCR contact (30).

It is unlikely that all potential self antigens can be presented in the thymus and evidence suggests that autoreactive T cells are present as a component of the normal peripheral T cell repertoire (46). It is possible that CLIP-specific CD4$^+$ T cells may escape negative selection and be present in the periphery. However, our own and previous findings have shown that mice immunized with murine CLIP fail to elicit T cell responses (22). This together with the observation that all of the T cell clones that we studied responded to both CLIP and p15–29 would argue against the existence of a population of peripheral T cells uniquely specific for CLIP.

The central implication of this paper is that T cell cross-reactivity between self and foreign antigens exists and that it may be possible to maintain immunological memory to foreign antigens by stimulation of specific memory T cells by self peptides displaying a degree of molecular mimicry. Whether this mechanism may contribute to the chronic nature of some diseases, including allergy requires further study. Our findings may also have an important consideration for the design of peptide vaccines for the therapy of immune disorders as well as for protection against infectious microorganisms.

Acknowledgements

We thank Drs D. Mathis and C. Benoist for the generous gift of the Ii deficient mice, and Professor B. Askonas and Dr M. Dallman for helpful discussions and reviewing the manuscript. This work was supported by grants from the Wellcome Trust and the Medical Research Council, UK.

Abbreviations

APC	antigen-presenting cell
CFA	complete Freund's adjuvant
CLIP	class II associated invariant chain derived peptide
Der p 1	*Dermatophagoides pteronyssinus* group 1 allergen
ER	endoplasmic reticulum
HDM	house dust mite
Ii	invariant chain
MBP	myelin basic protein
OVA	ovalbumin

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T-cell responses to allergens: epitope-specificity and clinical relevance

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REVIEW
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T-cell responses to allergens: epitope-specificity and clinical relevance

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Allergen-specific T cells play an important role in the pathophysiology of atopic allergies. Recently, cDNAs that encode many important allergens have been cloned and their amino acid sequences deduced, thus allowing the elucidation of the epitope-specificity of allergen-specific T cells. Here, Joost van Neerven and colleagues discuss the results of these studies, and the implications for the development of efficient strategies for specific immunotherapy.

In type I allergy there appears to be a breakdown of tolerance to innocuous proteins that is manifest by an immune response that causes a direct hypersensitivity reaction, local tissue damage and even systemic responses. In atopic patients, these symptoms are associated with high serum levels of allergen-specific IgE and eosinophilia, and are induced by a spectrum of type 2 cytokines produced by allergen-specific $CD4^+$ T cells. Specific inactivation of these cells, or the induction of T helper 1 (Th1) type cytokines, might directly interfere with the disease process. Therefore, allergen-specific T cells are obvious targets for intervention in atopic disease and a detailed characterization of the epitopes present on major allergens might lead to improved forms of therapy, using either synthetic peptides or recombinant allergens.

T-cell responses to allergens

Many allergen-specific human $CD4^+$ T-cell clones have been shown to resemble murine Th2 cells, producing large amounts of interleukin 4 (IL-4) and IL-5, but little or no IL-2 and interferon γ (IFN-γ) upon activation[1,2]. Th2-like cells have been identified in peripheral blood and lesions of atopic-dermatitis patients, in the nasal mucosa of rhinitis patients, and in the bronchial alveolar lavage (BAL) fluid of asthma patients; observations that highlight the involvement of Th2 cells in atopic diseases[1,3–5]. Figure 1 gives a schematic representation of the role of T cells in the allergic immune response.

Because activation of allergen-specific Th2-like cells seems to be causally related to the pathophysiology of allergic disease, information about the precise proteins they recognize and their T-cell epitopes is needed before allergen-derived synthetic peptides or recombinant allergens can be considered for immunotherapy.

Major allergens contain multiple T-cell epitopes

A large proportion of allergen-specific T cells are specific for major allergens[6–14], i.e. proteins that are recognized by serum IgE of more than 50% of allergic patients, and T-cell epitope mapping studies have focused on these molecules. The recent cloning and sequencing of several cDNAs that encode major allergens has allowed the identification of T-cell epitopes, as well as an estimation of their relative importance in inducing T-cell activation. Epitope mapping studies using long-term T-cell clones and lines, have found that all allergens studied to date, i.e. mite-, grass pollen-, tree pollen-, cat-, bee venom-, and chicken-derived allergens (see Table 1) contain multiple T-cell-activation-inducing epitopes dispersed throughout the molecule[9–12,14–17,19–33]. These epitopes are recognized by allergen-specific T cells isolated both from atopic patients and from healthy, nonatopic individuals[12,15,25]. In addition, T-cell clones with distinct epitope specificities for a given allergen can be isolated from a single atopic donor[9,10,16]. This suggests that certain epitopes are recognized in the context of different major histocompatibility (MHC) class II molecules[9,19], and that certain HLA molecules present multiple allergen-derived epitopes to the immune system. Furthermore, epitopes might exist that can only be presented by a single HLA-DR, DQ or DP molecule.

Polyclonal T-cell responses might give a better representation of the diversity of T-cell responses to allergens than responses measured with T-cell clones because a bias towards certain T-cell clones might be selected during the *in vitro* cloning procedure. As expected, polyclonal allergen-specific T-cell lines have been shown to recognize a broader range of T-cell epitopes, with T-cell lines of some atopic individuals recognizing up to six different epitopes within the same allergen[10,12]. Interestingly, when peripheral T cells from large panels of patients are tested for reactivity with peptides of major allergens, immunodominant regions can be identified[15,22–24,28,29,31].

MHC class II restriction and T-cell receptor usage of allergen-specific $CD4^+$ T cells

MHC restriction patterns of allergen-specific human T cells appear to be very diverse, as is T-cell epitope recognition. HLA-DRB1, HLA-DRB3, HLA-DP and HLA-DQ restriction has been demonstrated for several allergens[8,9,12,15,19,22,34–36]. Such diverse restriction patterns suggest that there is only a weak or no correlation between HLA

Table 1. Human T-cell epitopes of allergens

Allergen source	Allergen	Size[b]	T-cell epitopes	Individuals tested	T-cell source	Refs
Perennial allergens:						
Acarids						
Dermatophagoides	Der p 1	24 kDa, 222 aa	45–67, 94–104, 117–143	2	TCC	9
pteronyssinus	Der p 1		110–119, 110–131	1	TCL and TCC	20
	Der p 1		1–14, 1–56, 15–94, 57–130, 95–208, 188–222, 209–222	18	PBMC	21
	Der p 2	15 kDa, 129 aa	1–15, 11–24, 20–33, 29–42, 38–51, 47–60, 56–69, 92–105, 101–114, 116–129	5	TCL and TCC	10
	Der p 2		1–20, 11–35, 22–50, 36–60, 51–77, 61–86, 78–104, 81–96, 91–105, 87–112, 105–129	18	PBMC and TCC	11
	Der p 2		11–25, 16–31, 21–35, 22–40, 71–86, 81–96, 82–100, 111–129	1	TCL and TCC	22
	Der p 2		20–33	2	TCC	12
	Der p 2		1–15, 11–25, 21–35, 31–47, 41–55, 51–65, 61–75, 71–86, 81–96, 91–105, 101–115, 111–129	24[a]	PBMC	23
Mammals						
Felis domesticus	Fel d 1	17 kDa, 70+92 aa (dimer)	39–52, 53–66 (chain 1), 9–21, 22–35, 57–70 (chain 2)	4	TCL and TCC	19
	Fel d 1		1–17, 9–25, 18–32, 29–42, 37–55, 44–60, 56–70 (chain 1) 1–22, 12–33, 23–48, 34–59, 49–68, 60–82, 74–92 (chain 2)	53[a]	TCL	24
Seasonal allergens:						
Trees						
Betula verrucosa	Bet v 1	17 kDa, 159 aa	2–16, 11–22, 61–72, 77–88, 85–96, 113–124, 145–156, 147–158	6	TCC	17
	Bet v 1		1–16, 27–40, 35–48, 75–92, 77–92, 93–110, 141–156	2	TCC	16
	Bet v 1		1–16, 11–26, 61–76, 63–78, 65–80, 75–90, 77–92, 95–110, 97–112, 111–126, 113–128, 127–140, 141–156	9	TCC	25
	Bet v 1		1–15, 8–23, 19–33, 29–43, 46–63, 58–73, 65–79, 73–87, 82–96, 90–104, 117–131, 99–113, 126–140	3	TCL and TCC	S.H. Sparholt et al, unpublished
Cryptomeria japonica	Cry j 1	41–45 kDa, 353 aa	327–346, 337–353	1	TCC	26
Grasses						
Lolium perenne	Lol p 1	34 kDa, 240 aa	191–210	1[a]	TCC	27
	Lol p 1		Several[c]	6[a]	PBMC	28
	Lol p 1		1–20, 11–30, 21–40, 31–50, 41–60, 50–70, 71–90, 91–110, 101–120, 111–130, 121–140, 131–150, 141–160, 151–170, 171–190, 181–200, 191–210, 221–240	8[a]	TCL and TCC	14
	Lol p 1		Several[c]	6[a]	PBMC	29
Phleum pratense	Phl p 1	34 kDa, 240 aa	22–36, 25–39, 34–45, 70–84, 73–84, 91–102, 97–111, 91–102, 100–114, 109–123, 121–134, 127–138, 130–141, 142–155, 157–168, 169–183, 211–225, 226–240	9	TCC	30
Poa pratensis	rKBG60	28 kDa, 268 aa	peptide 5, 99–118, 109–128, 149–168, 159–178, 169–188, 199–218, 219–238, 229–248, 239–258, 249–268	13[a]	PBMC	31
Venom allergens:						
Insects						
Apis mellifera	Api m 1 (PLA_2)	19 kDa, 134 aa	50–69, 83–97	1	TCL	32
(Honey bee)	Api m 1 (PLA_2)		45–62, 74–91, 76–93, 81–92, 81–98, 107–124, 111–128, 113–124, 114–131	40[a]	PBMC and TCC	15
Food allergens:						
Birds						
Chicken	Ovalbumin	43 kDa, 385 aa	1–33, 198–231, 201–213, 261–277	4	TCC	33

Abbreviations: aa, amino acids; PBMC, peripheral blood mononuclear cells; PLA_2, phospholipase A_2; TCC, T-cell clone; TCL, T-cell line.
[a] Even though the presence of several T-cell epitopes was described, T-cell epitopes that were recognized by >50% of all individuals tested have been identified in these studies.
[b] Sizes are shown as SDS-PAGE mobility of the native protein (in kDa) and as number of amino acids (based on the recombinant sequence).
[c] These papers describe reactivity with several peptide pools. Exact amino acid sequences are not clear.

alleles and IgE responsiveness. Correlations between HLA and IgE responsiveness have been described (see Table 2) but p-values and relative risks appear to be high only for the smallest allergens, which presumably contain limited sets of T-cell epitopes owing to their size[37–43]. In addition, because allergenic extracts are generally complex mixtures of many allergens, a correlation between HLA and one of these allergens does not strongly affect IgE responsiveness to an allergen extract.

In some immunological diseases there is an oligoclonal expansion of T cells that express a limited number of T-cell receptor (TCR) variable (V)β-gene products[44,45]. If allergen-specific T cells with a limited use of TCR V-gene products play a role in allergic responses, the possibility exists for immunotherapy specifically targeted at the TCR expressed on these cells. However, as expected from the HLA restriction and epitope diversity, human allergen-specific T-cell clones use a diverse set of T-cell receptor Vβ- and Vα-gene segments[9,10,30,46–48]. Although only a small number of T-cell clones have been tested in these studies, no biased TCR V-gene product usage could be demonstrated, thus complicating the issue of TCR-directed intervention.

Conversely, the results of Mohapatra *et al.* suggest that the diversity of the TCR Vα- and joining (J)α-gene segment usage of Lol p 1-specific T-cell clones appears to be limited[49], whereas it was shown in another study that T-cell responses to Der p could be inhibited by TCR Vβ-derived synthetic peptides: indicating that TCR usage by allergen-specific T cells is restricted in some individuals[50].

A recent study showed that polyclonal populations of T cells, expressing almost the full range of TCR Vα- and TCR Vβ-gene segments, are present in BAL fluid and peripheral blood of allergic-asthma patients[51]. However, after exposure to allergens, an oligoclonal T-cell expansion could be observed in BAL fluid, but not in the peripheral blood, suggesting that allergen-specific T cells are expanded or recruited in lung tissue after allergen exposure[51].

TCR usage has also been employed to estimate the longevity of allergen-specific T cells. Wedderburn *et al.* have reported that T-cell clones with identical TCR V and J regions can be isolated from the same patients after an interval of six years[52]. These findings are consistent with data from Ebner *et al.*, who reported that epitope-specificity of Bet v 1-specific T cells from a birch-pollen-allergic patient was unchanged when T cells were isolated from the same patient after a two-year interval[16].

Importance of isoallergenic variation for the design of immunotherapy

It is becoming increasingly clear that many isoallergenic forms of major allergens exist in nature[53]. These isoallergens differ from one another in one to several amino acids[18,27,54,55]. Some isoallergens have a very high degree of identity with homologous allergens in related species, which might explain the high degree of crossreactivity at the IgE level, as well as at the T-cell level.

As a result of structural homology, major-allergen-specific T cells might crossreact with allergens from different species within the same genus, as shown for house dust mite Der f-specific T-cell clones and lines that crossreact with house dust mite Der p (Refs 7, 35). A similar phenomenon can be observed among grass pollen group 1 allergen-specific T cells[30] and tree pollen group 1 allergen-specific T cells (S.H. Sparholt *et al.*, unpublished) that crossreact with group 1 allergens from different genera of grasses and trees. At least a part of the latter crossreactivities must result from isoallergenic variation since some natural Bet v 1 or Phl p 1-specific T-cell clones were shown to crossreact with group 1 allergens from other families, but did not react with recombinant Bet v 1 or Phl p 1, representing single isoforms.

The existence of many isoallergens has several implications for possible use of peptides in immunotherapy: T-cell epitopes identified by using T cells that react with recombinant allergens (and their peptides) might represent only a proportion of the repertoire of allergen-specific T cells present in the human body. Studies by Ferreira *et al.*[56] and by S.H. Sparholt *et al.* (unpublished) clearly show that multiple isoallergens are involved in the sensitization to Bet v 1, the major birch-pollen allergen. Some of the isoforms studied have complete T-cell-activation capacity, but fail to bind IgE *in vitro*, and induce very weak skin prick tests[56]. Such isoforms can be considered for immunotherapy, since they might be safer than natural extracts, owing to decreased IgE binding and the decreased risk

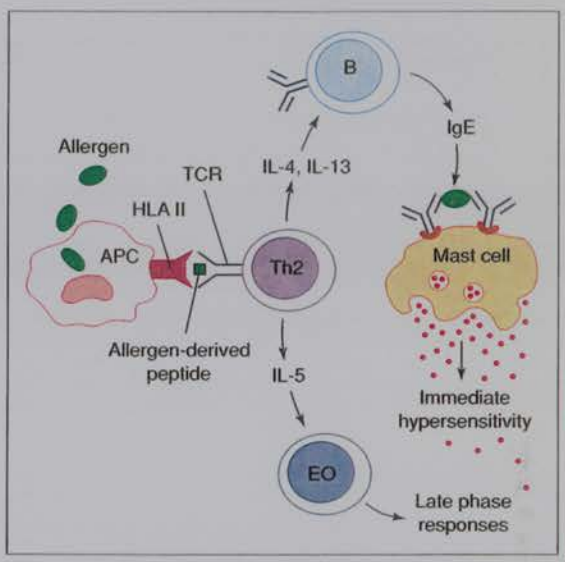

Fig. 1. *The role of CD4⁺ T cells in the allergic immune response. Upon entry into the human body, the allergen is taken up and processed by an APC. The presented allergen-derived peptide is recognized by an allergen-specific Th2-like cell that produces IL-4, IL-5 and IL-13. IL-4 and IL-13 induce the isotype switch to IgE in B cells. IgE binds to the high affinity Fcε receptor on mast cells, which degranulate upon Fcε-receptor crosslinking, resulting in the immediate Th1-type response. IL-5 produced by the Th2-like cells plays a role in the differentiation and survival of eosinophilic granulocytes, which are responsible for the late-phase response (especially in allergic asthma). Abbreviations: APC, antigen-presenting cell; EO, eosinophilic granulocytes; IL-4, interleukin 4; TCR, T-cell receptor; Th2, T helper 2 cell.*

Table 2. Associations between HLA and IgE responsiveness to single allergens

Allergen	Molecular mass (kDa)	HLA-DR	IgE^+ (%)	IgE (%)	p-value	Relative risk
Amb a 5	5	DR2/Dw2	100	24	<0.0001	65
Lol p 2	11	DR3	47	15	0.007	5.3
Lol p 3	11	DR3	43	18	0.007	3.5
Lol p 3	11	DR3	57	7	0.00001	18
Amb a 6	11.5	DR5	85	14	0.0000007	35
Amb a 6	11.5	DR5	40	6	0.001	23
Alt a 1	14	DR4	26	16	0.006	1.9
Der p 2	15	DR3	19	16	0.034	>1
Bet v 1	17	DRw52a/c	62	33	<0.02	2.5
Bet v 1	17	DRB3*0101	51	30	0.02	2.5
Fel d 1	17	DR1	16	9	0.02	2.0
Der p 1	24	DR3	16	17	0.078	< 1
Lol p 1	34	DR3	36	7	0.01	7.3
Lol p 1	34	DR3	33	14	0.01	3.1

for induction of anaphylactic reactions. However, their potential use might be limited since not all T-cell specificities will be present on these isoforms.

Modulation of allergic responses using synthetic peptides

In order to evaluate the potential of synthetic peptides for immunotherapy, both *in vitro* and *in vivo* experimental systems have been used. Using these models, the ability of allergen-derived synthetic peptides to inhibit cellular responses and humoral responses to allergens has been studied, providing evidence that allergen-specific immune responses can be inhibited.

It has been demonstrated that the incubation of human influenza-specific T-cell clones with supraoptimal concentrations of influenza peptide in the absence of antigen-presenting cells (APCs) resulted in a state of nonresponsiveness (or anergy), during which no proliferative responses were observed – even after subsequent stimulation of the cells with the same peptide in the presence of APCs (Ref. 57).

More recently, using the same experimental conditions, it has been shown that anergy can also be induced in allergen-specific Th2 clones following stimulation with allergen-derived peptides[58–60]. After initial activation, accompanied by the production of large amounts of cytokines, including IL-4, IL-5, IL-13, granulocyte–macrophage colony-stimulating factor (GM-CSF), and tumor necrosis factor α (TNF-α) (S. Fasler and H. Yssel, unpublished), Der p 1-specific Th2 clones become unresponsive to further stimulation with Der p 1-pulsed APCs (Refs 58, 59). Interestingly, stimulation of the anergic T-cell clones with a combination of phorbol ester and calcium ionophore resulted in the production of normal levels of these cytokines[58,59], suggesting that peptide-mediated nonresponsiveness might result, in part, from impaired signalling through the TCR/CD3 complex, independent from the expression of this complex on the surface of the cells[59].

However, information about signalling pathways in human anergic T cells is still limited. It has been reported that the anergic state induced in mouse T-cell clones following stimulation with altered peptides is characterized by a unique pattern of phosphorylation of the TCRζ chain, as well as a failure of the protein kinase Zap-70 to associate with this chain[61]. It has yet to be established whether similar observations can be made in peptide-mediated anergy induced in human T-cell clones. Importantly, in contrast to Der p 1-specific T-cell clones optimally stimulated with antigen-pulsed APCs, anergic Th2 clones failed to give help to B cells for the synthesis of IgE, even in the presence of exogenous IL-4 or IL-13, despite comparable levels of expression of the costimulatory CD40 ligand on both active and anergic T-cell populations[59].

The activation of allergen-specific T cells and immunoglobulin synthesis in mice can also be inhibited *in vivo* by intra-nasal, oral or subcutaneous administration of peptides[60,62–64]. Naive mice that were pretreated with the dominant peptide of Fel d 1 or Der p 1 did not develop an immune response when challenged with native Der p 1 or Fel d 1. In addition, ongoing immune responses to native Der p 1 and Fel d 1 could be substantially downregulated by peptide treatment. This could also be achieved using minor epitopes of Der p 1 that are recognized when mice are immunized with these peptides but not when they are immunized with whole protein[64]. It should be noted that administration of peptides results in inhibition of responses to the entire allergen, indicating that not all allergen-derived T-cell epitopes have to be present to obtain *in vivo* tolerance to allergens.

Can synthetic peptides of allergens be used for immunotherapy?

Allergen extracts have been extensively characterized and standardized over the past decades, however, allergen immunotherapy has hardly changed since its introduction by Noon in 1911 (Ref. 65). During immunotherapy, gradually increasing doses of complete allergen extract are injected, resulting in a reduction of clinical symptoms of allergy. T cells are believed to be the target cells that are affected by successful immunotherapy but there is no obvious evidence concerning the mechanism (see Fig. 2).

As a result of the abundance of human T-cell-activation epitopes on allergens (see Table 1), and the problems posed by the occurrence of isoallergenic variation, it is clear that immunotherapy might have an optimal efficacy when natural allergen extracts, and not peptides or recombinant allergens, are used. Cross-epitope inhibition can be demonstrated in experimental models[62,63], although as yet, there is no evidence in humans to support the contention that vaccination with a single peptide will downregulate the response to an intact protein.

However, adverse reactions occur occasionally during specific immunotherapy with native allergens as a result of binding of injected allergens to allergen-specific IgE bound to mast cells. This

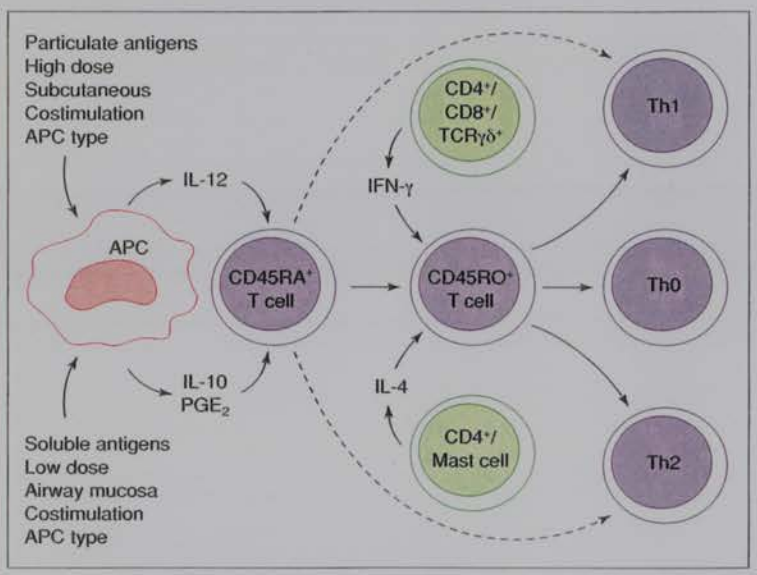

Fig. 2. *Factors and cells involved in the generation of Th2, Th0 and Th1 responses. APC-derived factors, T-cell derived cytokines, APC type, and the dose and administration route of allergens can strongly influence the outcome of an allergen-specific T-cell response. During natural sensitization, low doses of allergens are absorbed in the airway mucosa, leading to Th2-type responses in atopic individuals. During specific immunotherapy, high doses of allergens are injected subcutaneously over 3–5 years, resulting in a bias for the production of Th1-type cytokines[71–73]. Several novel immunotherapy strategies are under development that might specifically interfere with the outcome of allergen-specific T-cell responses. The use of particulate antigens (to select for IL-12-producing professional APCs), naked DNA vaccination (to induce IFN-γ-producing HLA class I-restricted T cells), Th1-biasing adjuvantia, and high doses of peptides (high dose without IgE related side-effects) or recombinant allergens (easier to standardize than complex allergen extracts) might result in a bias for the production of Th1-type cytokines. However, oral, nasal, or sublingual delivery of allergens, and high doses of peptides might directly induce tolerance in allergen-specific Th2-like cells. Abbreviations: APC, antigen-presenting cell; IFN-γ, interferon γ; IL-10, interleukin 10; PGE_2, prostaglandin E_2; Th1, T helper 1 cell.*

can be avoided if allergen-derived synthetic peptides are used instead of whole allergen extracts. Binding of allergen-specific IgE to allergen-derived peptides is strongly reduced[66], whereas the desired effects on T cells are unaltered. Therefore, the safety of immunotherapy can increase when peptides or low IgE-binding recombinant allergens are used. The answers to these problems will have to await results of clinical trials with peptides or recombinant allergens. So far, a trial with bee venom phospholipase A_2-derived peptides has shown good clinical efficacy with no adverse reactions[67], and another trial using Fel d 1-derived peptides is currently under way[68].

An important question that these clinical studies will have to answer is whether the inhibition of immune responses to major allergens will be sufficient to silence allergic responses to other proteins present in the allergen extract, or whether the specificity of the allergic response will shift to minor allergens that can also be recognized by T cells[7,19,69]. This might be expected from the work of Gammon *et al.* who showed that T cells with specificities for minor epitopes of antigens can escape tolerance induction[70]. Several studies have indicated that a shift in the production of Th2 to Th1 cytokines takes place in successful immunotherapy[71–73]; this might result from the activation of IFN-γ-producing, allergen-specific $CD8^+$ or $TCR\gamma\delta^+$ cells that inhibit IgE synthesis, as described in animal models[74,75]. If this turns out to be the mechanism underlying successful immunotherapy, careful selection and combination of peptides of several major allergens might be the most crucial step in designing peptide therapies. However, the high degree of isoallergenic variation of some allergens, such as tree pollen and grass allergens might favour approaches that are based on the use of natural allergenic extracts that are degraded to peptides[76], or on the use of recombinant T-cell epitopes that do not bind IgE, as described by Rogers *et al.*[18].

Another strategy for optimizing allergy immunotherapy is directed at finding ways to change the cytokine profiles of allergen-specific $CD4^+$ T cells. The crudest method would be to co-inject a protein extract (e.g. purified protein derivative), cytokine (IL-12, IFN-γ), or an IL-12-inducing factor, in combination with allergen extracts in order to shift the T-cell response towards a Th1 spectrum. T cells that maturate in this artificially created microenvironment will preferentially become T cells with a Th0- or Th1-type cytokine profile. Alternatively, other approaches might be aimed at inserting allergens in attenuated live, bacterial or viral vectors, that themselves evoke strong Th1-type responses. In addition, studies on the role of APCs in the generation of Th2 vs. Th1-type responses by the production of soluble mediators such as prostaglandin E_2 or IL-12, respectively, might yield information on the underlying mechanism of the *in vivo* outgrowth of T cells with biased cytokine production profiles[77].

Concluding remarks

The increasing knowledge of the role of the immune system in the pathophysiology of atopic disease has revealed new possibilities for designing immunotherapeutic approaches. The future course of specific immunotherapy as treatment for allergic diseases will to a large extent be dependent on the results of clinical trials that use synthetic peptides, recombinant allergens, degraded allergenic extracts, or modified allergens that selectively tolerize Th2-type or activate Th0-type or Th1-type responses to allergens.

DNAX Research Institute of Molecular and Cellular Biology is supported by Schering-Plough Corporation.

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1.3 ANALYSIS OF RESTRICTION SPECIFICITY BY MAJOR HISTOCOMPATIBILITY COMPLEX CLASS II PROTEINS

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Immuno-genetics

Human Helper T-Cell Clones That Recognize Different Influenza Hemagglutinin Determinants Are Restricted by Different HLA-D Region Epitopes

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Abstract. Human T-lymphocyte clones (TLCs) were generated against the hemagglutinin (HA) of A/Texas/1/77 influenza virus by limiting dilution. TLCs were then screened for antigen specificity on chemically synthesized peptides representing the HA1 molecule. It has been hypothesized that different T cells that recognize the identical antigenic determinant are controlled by (restricted by) the same class II epitope. Two TLCs, HA1.4 and HA1.7, both recognized the same HA peptide and in proliferation studies exhibited identical restriction patterns. Two other clones, HA 1.9 and HA 2.43, recognized different HA determinants and also had distinct restriction patterns. Proliferation inhibition studies with monoclonal antibodies against human class II molecules demonstrated three unique patterns of blocking with the clones, suggesting that clones may be restricted to a unique class II epitope depending on the HA determinant recognized. These data can be interpreted as supporting the argument that human immune responses to influenza hemagglutinin are under *Ir* gene control exerted at the level of the viral antigenic determinant recognized in association with particular *D*-region restricting elements. The determinant selection and clonal deletion theories are compared for their capacity to best explain these findings.

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Abbreviations used in this paper: ^{3}HTdR, tritiated methyl thymidine; MHC, major histocompatibility complex; HLA, human MHC; PBLs, peripheral blood lymphocytes; APCs, antigen-presenting cells; TLCs, T-lymphocyte clones; TCGF, T-cell growth factor; MoAbs, monoclonal antibodies.

Introduction

Class II molecules are thought to be immune response (Ir) gene products (Berzofsky 1980). Mouse studies have shown that both the *I-A* and *I-E* subregions (Klein et al. 1981) control the ability to respond to particular antigens including such diverse materials as random synthetic peptides (McDevitt and Benacerraf 1969), sperm whale myoglobin (Berzofsky et al. 1982), cytochrome C (Heber-Katz et al. 1982), and DNP-ovalbumin (Sredni and Schwartz 1980). The concept emerging from these investigations is that a particular *Ir* gene controls immune responses to a single antigenic determinant, probably by influencing the way in which the T cell recognizes antigenic structures. One interpretation of such experiments depends on the ability of antigen-presenting cells (APCs) to display antigen on their surfaces in a form immunogenic to T cells (Rosenthal et al. 1981, Benacerraf 1978). APCs lacking appropriate *Ir* genes (and presumably the appropriate class II molecules, therefore) are unable to present antigen *to specifically primed T-lymphocytes*. An alternative hypothesis suggests that *Ir* gene phenomena reflect deletion from the T-cell repertoire of those clones which are capable of recognizing a given antigen in the context of a particular restricting element; where this deletion occurs is not entirely clear (Schwartz 1978, Ishii et al. 1983). Typically, experiments examining the above questions require that antigen be presented to primed T cells by allogeneic or semiallogeneic APCs. A problem with this approach, in the mouse and especially in man, is that even primed T-lymphocyte populations contain cells which recognize allogeneic (nonself) class II antigens and thereby obscure responsiveness to specific antigen (self + X). Another aspect of this same problem is that the human major histocompatibility complex (MHC) is incompletely understood so that while two individuals may share some identical *D*-region antigens, there may exist other regions or loci contributing to class II antigen disparity and thus, high background alloreactivity (Bergholtz et al. 1980, Kurnick et al. 1981). This consideration further muddles an already complex interpretation of the experimental data.

We have used T-cell clones to investigate the nature of *Ir* gene control of cellular immune responses to influenza virus antigens. The clones are antigen-specific (Lamb et al. 1982a, b, c) and restricted by antigens encoded in the *HLA-D* region (Eckels et al. 1982, 1983a, b; Lamb et al. 1982a) in that they recognize only components of influenza A/Texas, not B/Singapore, and respond only when antigen is presented by APCs bearing class II molecules common to both the T-lymphocyte clones (TLC) and APC. We have posed the question of whether clones which recognized the same antigenic determinant were also restricted by identical human class II molecules (*HLA-D* region antigens) or *Ir* gene products. We report here on four hemagglutinin specific clones that recognize three distinct HA determinants and which are restricted by at least three different epitopes associated with the *HLA-D* region. When monoclonal antibodies (MoAbs) specific for human class II molecules were used to block TLC-APC interactions, three different blocking patterns were obtained, that is, two clones which apparently recognize the same HA determinant had identical patterns of blocking with the MoAbs on two different, allogeneic APCs, suggesting that these clones may be restricted by the same epitope. These results are compatible with the concepts that TLCs recognizing different antigenic determinants can be controlled by distinct *Ir* genes and that T-cell

recognition as reflected in *Ir* gene phenomena is exquisitely sensitive to subtle differences in the combination or interaction of antigenic determinants with their corresponding restricting epitope.

Materials and Methods

Clones. Peripheral blood lymphocytes (PBLs) (2.5×10^5/ml) were primed under optimal conditions with the isolated hamagglutinin (HA) of A/Texas/1/77/x-49 influenza virus (0.1 μg/ml) or whole virus (5 HAU/ml). After 6 days, responding cells were cloned by limiting dilution (0.3 cells/well) in the wells of Falcon Microtest II trays, each containing 10^4 autologous PBLs (30 Gy) and antigen in 20-μl tissue culture medium with 20% TCGF. TLCs were successively expanded, maintaining the same proportion of T-cell growth factor (TCGF), feeders and antigen (clone split to 2×10^5/ml) and have exceeded 10^9 viable, functional cells. TLCs could be frozen in standard protocols and recovered for later use. These methods have been extensively described elsewhere (Lamb et al. 1982a, 1983a, b, Eckels et al. 1982).

Assay conditions. TLCs were assayed for proliferation as indicated by incorporation of ^{3}HTdR after 72 h. Clones at $5–10 \times 10^3$/well were cultured with $2.5–5.0 \times 10^3$ irradiated histocompatible and histoincompatible PBLs or with 5×10^3 irradiated E^- (T-cell depleted) APCs; optimized antigen concentrations were used as indicated (A/Texas, B/Singapore/222/79, A/Japan/170/62 and A/Japan/305/57 at 2.5–20 HAU/ml; HA from A/Bangkok/1/79 and synthetic peptide at 0.25–2.0 μg/ml). EBV-transformed lymphoblastoid cell lines were used at 2.5×10^4/well (60 Gy) with optimal antigen concentrations. Further details can be obtained from Lamb and co-workers (1982b, c). Synthetic peptides of the HA1 molecule were kindly provided by Drs. N. Green and R. Lerner, Scripps Institute, San Diego, California.

HLA phenotyping was performed as previously described (Eckels and Hartzman 1982, Amos and Kostyu 1980).

Monoclonal antibodies against human class II molecules were analyzed for their capability of blocking TLC proliferation. MoAb (approximately 0.2–50 μg/ml) was added to optimized cultures and was present throughout the 72-h culture period. The MoAbs used in this study included the DR-specific SG157 (Goyert et al. 1982a), DS-specific SG171 (Goyert et al. 1982b), DC-specific SG465 and SG520, (Goyert and Silver 1983), kindly provided by Drs. S. Goyert and J. Silver, the DR-specific monoclonal antibodies IIIE3 and IVG1 (Hurley and Nunez 1982) which were generously provided by Drs. C. Hurley and D. Capra as well as Q2/70 and Q5/6 (Quaranta et al. 1981) which react with isolated class II beta chains and were kindly provided by Dr. V. Quaranta.

Results

TLC specificity and function. The TLCs used in this study have been extensively characterized. In summary, the TLCs HA1.4, HA1.7, HA1.9, HA2.43, FL2.8, and FL2.20 have been shown by fluorescence-activated cell sorter analysis to have the following phenotype: Leu-1$^+$, T3$^+$, T4$^+$, Leu-7$^-$, T8$^-$, T10$^=$, T11$^+$, class I$^+$, class II$^+$, sIg$^-$ (data not shown). Furthermore, HA1.4, HA1.7, HA1.9, and FL2.20 have been observed to induce the release of influenza virus-specific antibodies from autologous B-lymphocytes (Lamb et al. 1982d, 1983a, b; J.R. Lamb, personal communication). All except HA1.9 and FL2.8 secrete gamma interferon when induced by specific antigen or T-cell mitogens (S. Jacobson et al., manuscript in preparation). The antigen specificities of the HA-specific clones are shown in Table 1. Note that (1) TLCs HA1.4, HA1.7 and HA2.43 recognized peptide 20 of the HA1 molecule as well as purified HA from A/Bangkok, (2) HA2.43 recognized A/Japan/305, and (3) HA1.9 failed to respond to peptide 20 although it recognized and responded to A/Japan/170 and A/Texas. Similar results have been reported by

Table 1. Antigen specificities of HA-specific TLCs

Antigen	Range of TLC responses (cpm)*			
	HA1.4	HA1.7	HA1.9	HA2.43
A/Texas/1/77/x-49 (intact virus)	926–8 062	219–8 284	257–4 325	152–1 094
Purified HA (A/Texas; H3)†	+	+	+	+
Purified HA (A/Bangkok; H3)	8 938–23 999	4 669–25 191	129–852	1 132–5 255
A/Japan/170/62 (intact virus)	1 419–2 725	501–739	31 052–49 842	367–820
A/Japan/305/57 (intact virus)†	–	–	nt	+
Peptide 20 (aa 306–330)	26 325–37 844	22 728–32 047	76–136	4 024–6 879
B/Singapore/222/79 (intact virus)	70–112	56–74	42–209	46–87

* TLC was used at 10^4/well, autologous PBLs as a source of APCs at 5×10^4/well with antigen as follows: A/Texas 5–40 HAU/ml; purified HA from A/Bangkok 0.25–2.0 μg/ml; A/Japan/170 1:500 to 1:4 000 dilution; p20 p.25–2.0 μg/ml; B/Singapore 5–40 HAU/ml. Note significantly high backgrounds in the presence of A/Japan/170 due to backstimulation from T-cells in PBL population (cf. Lamb et al. 1982b).

† Antigen previously tested on these TLCs and is no longer available. Therefore, results are summarized from Lamb et al. 1982c.

Lamb and co-workers (1982c) and suggest that HA1.4 and HA1.7 may recognize the same antigenic determinant which may be distinct from that recognized by HA2.43 which is in turn distinct from that recognized by HA1.9. Thus, the four hemagglutinin specific TLCs were capable of recognizing three different HA determinants. Based on the above data, the following postulate was suggested: Clones recognizing three distinct determinants should have three distinct restriction patterns if distinct *Ir* gene products control such responses.

Restriction patterns of HA-specific TLCs. Eleven different TLCs were analyzed for responsiveness on a panel of histo*compatible* and histo*incompatible* PBLs. The panel included irradiated cells from 84 individuals representing 158 unrelated haplotypes, as well as 16 EBV-transformed lymphoblastoid B-cell lines (LCLs). Representative data are provided from 31 panel members due to space considerations. However, the statistics included are based on the full panel. The current HLA phenotypes of panel members used herein are shown in Table 2. The response patterns of alloreactive (AL14.12, AL14.65, AL14.71, S3.11 and S3.233) and antigen reactive (FL2.20, FL2.38, HA1.4, HA1.9, and HA2.43) clones are shown in Table 3. The Dw1/DR1 alloantigenic complex is defined by TLCs AL14.12, AL14.65 and AL14.71. DR3-associated alloantigens are detected by the TLCs S3.11 and S3.233. Note that not all $DR1^+$ or $DR3^+$ stimulating cells were capable of eliciting a response from all of these alloreactive clones. The response patterns of two virus-specific clones (FL2.38 and FL2.20) with restriction associated with DR1 and DR3 antigens, respectively, are also presented for comparison. It is not known which viral subcomponents FL2.20 and FL2.38 recognize.

Of the four HA-specific TLCs, HA1.4 and HA1.7 recognized the same peptide fragment and were also restimulated by virus presented on the same APCs: Both TLCs were positive with 13/17 $DR1^+/Dw1^+$ APCs, 9/25 $DR4^+$ APCs and 1/46 $DR1^-/Dw1^-$, $DR4^-$ APCs. No clear statistical association was evident between

positive responses by HA1.4 and HA1.7 and any other known *HLA* allele. In contrast, the TLC HA1.9 recognized a different determinant on the HA molecule and also had a different restriction pattern, recognizing virus presented by 7/15 $DR1^+/Dw1^+$ APCs. Finally, HA2.43 recognized a hemagglutinin determinant present in peptide 20 and A/Japan/305 as well as A/Bangkok HA unlike the other three TLCs and HA2.43 also exhibited a third restriction pattern in that it was clearly restimulated only by autologous APCs and marginally by 2/15 unrelated $DR1^+/Dw1^+$ APCs.

Lymphoblastoid cell lines from homozygous typing cell donors were used to present viral antigens to HA1.4 and HA1.7 (Table 4). The LCLs were kindly provided by Dr. N. Suciu-Foca of Columbia University, New York. As might be expected, TLCs HA1.4 and HA1.7 responded to virus presented by AR, a $Dw1^+$

Table 2. HLA phenotyping of cell panel

Cell	*HLA* loci								
	A	*C*	*B*	*w4/w6*	*D*	*DR*	*MB*	*MT*	*SB*
0006	1,*	7,	8,	6,	3,0*	3,0	2,0	2,0	1,4
0022	2,2	5,5	44,44	4,4	4,4	4,4	3,3	3,3	4,4
0127	1,32	,	14,8	,6	3,7	3,7	2,0	2,3	1,2
0144	1,	7,	8,	6,	1,3	1,3	1,2	1,2	2,4
0500	2,3	4,4	35,35	6,6	5,1	5,1	3,1	2,1	4,4
0633	3,3	8,0	14,41	6,6	0,0	1,4	1,0	1,0	4,4
0634	3,11	0,0	41,52	6,4	4,6	4,61	0,1	0,1	4,2
0640	25,32	,	39,8	4,6	DB3,DB3	4,4	3,	,	3,3
9030	1,3	,	8,7	6,6	7,2	7,2	0,1	0,1	4,4
0938	24,2	2,5	27,44	4,6	4,5	4,5	0,3	3,2	6,2
0954	30,29	6,3	13,60	4,6	1,LD40	1,4	1,0	1,0	0,3
0957	2,2	2,8	41,35	6,6	3,8	3,8	2,3	2,2	1,4
1010	2,24	3,6	62,45	6,	4,BSK	4,9	3,0	3,0	1,4
1011	28,23	7,0	18,49	4,6	1,0	1,4	1,3	1,3	4,6
1014	1,23	4,	53,45	4,6	2,0	2,3	1,0	1,2	1,3
1023	2,31	4,3	51,60	4,6	1,10	1,4	1,0	1,3	3,4
1042	1,28	6,7	57,18	4,6	7,0	7,4	0,0	3,0	4,6
1053	28,3	6,3	58,91	4,6	0,0	3,7	2,0	2,3	1,0
1060	2,24	5,	44,8	4,6	4,3	4,3	3,4	3,2	4,4
1061	11,21	,1	7,27	6,4	2,3	2,3	1,2	1,2	4,4
1078	1,3	7,7	8,7	6,6	3,5	3,5	2,3	2,2	4,2
1279	1,26	1,5	27,44	4,4	1,0	,	,	,	2,4
1285	,30	,3	,60	,	,LD40	,4	,	,	1,6
1286	,	,	,	,	4,0	,	,	,	4,0
1290	,	,	,	,	1,	,	,	,	4,0
1291	,	,	,	,	1,	,	,	,	4,7
1298	33,2	8,	14,55	,	1,TB24	1,2	1,1	1,	4,7
1528	1,3	3,7	55,7	6,6	4,2	4,2	2,1	,1	2,0
1531	2,24	0,3	52,62	4,6	0,1	2,1	1,1	1,1	2,0
1651	2,31	,8	8,14	6,6	6,7	61,7	1,2	2,3	4,2
2000	,	,	,	,	,	2,3	,	,	1,

* Zero indicates that the specificity was typed for but no assignment could be made. Blank indicates that the specificity was not typed for.

Table 3. Restriction patterns of human T-lymphocyte clones

Cells	HLA-DR	HLA-D	AL14.71	AL14.12	AL14.65	S3.11	S3.233	FL2.38
1298	1,2	1,TB24	46	21	1925	21	25	1275
1291	−,−	1,−	23	12	9271	27	19	1794
633	1,4	−,−	183	43	4055	37	34	2429
500	1,5	1,5	+	+	+	NT	NT	38103
1290	−,−	1,−	5059	8507	4709	28	15	2183
954	1,4	1,LD40	6986	2997	2404	24	17	995
1023	1,4	1,10	4050	3263	2655	37	19	1877
1011	1,4	1,−	9049	1875	3440	674	230	8764
1279	−,−	1,−	10863	1774	5549	133	155	7856
1531	1,2	1,−	3515	8457	2613	27	15	2658
144*	1,3	1,3	13833	3392	9469	2465	2929	13864
6	3,−	3,−	30	45	28	7304	3289	237
1053	3,7	−,−	18	20	35	1123	2483	0
127	3,7	3,7	25	27	19	527	1436	5
957	3,8	3,8	25	35	34	3852	862	21
2000	−,−	2,3	27	35	23	1652	1282	20
1286	−,−	4,−	23	79	41	133	2093	39
1078	3,5	3,5	17	24	21	242	281	109
1014	2,3	2,−	27	24	36	16	23	58
22	4,4	4,4	37	25	31	26	19	37
1010	4,9	4,BSK	201	218	50	799	29	0
1528	2,4	2,4	18	23	28	39	20	31
634	4,6.1	4,6	−	−	−	NT	NT	352
640	4,4	DB3,DB3	NT	NT	NT	NT	NT	48
938	4,5	4,5	51	39	37	15	19	19
1042	4,7	−,7	NT	NT	NT	NT	NT	892
1060	4,3	4,3	NT	NT	NT	NT	NT	2171
1285	4,−	LD40,−	35	25	218	17	23	34
930	2,7	2,7	62	49	44	9	24	15
1061	2,3	2,3	NT	NT	NT	NT	NT	92
1651	6,1.7	6,7	NT	NT	NT	NT	NT	53

* Autologous clone donor.
+/−, positive or negative in previous testing.
NT, not tested.
Underline indicates positive response.

LCL. Additionally, the 7618 LCL (Dw4$^+$) was able to present antigen to HA1.4 and HA1.7 while the 7337 LCL (DR4$^+$/Dw10$^-$) failed to present. Thus, these results confirm the conclusion and that the restriction element recognized by HA1.4 and HA1.7, though common to some Dw1$^+$ or Dw4$^+$ cells, is separate from the molecule or epitope expressing the DR determinant.

To summarize, three distinct patterns of restriction were observed for the HA-specific clones: HA1.4 and HA1.7 both recognized the same antigenic fragment (peptide 20) and showed concordant restricted patterns of responsiveness; the restriction patterns for HA1.9 and HA2.43 were different from HA1.4 and HA1.7 and were also quite different from one another which again may correspond to the fact that they recognized distinct HA determinants. The statistics of association between restriction responses and known *HLA-D* region alleles were inconclusive

FL220	HA1.4	HA1.7	HA1.9	HA2.43
25	16	12	3060	15
45	24	17	5561	26
37	27	24	2335	24
243	47198	33253	NT	NT
22	4776	3008	49	170
24	4661	3426	381	272
22	3705	2121	66	49
106	11286	9032	2434	905
31	8867	9556	2368	731
38	3736	3151	115	135
4463	15519	13100	10141	2120
8430	45	25	23	65
1424	122	37	47	32
563	20	26	70	38
75	15	6	23	19
2317	29	24	17	18
403	183	215	21	26
1111	15	16	28	16
35	14	22	54	42
22	1336	1209	17	24
281	7231	6854	153	300
29	2328	478	29	38
13	7	14	NT	NT
0	41	0	NT	NT
32	99	43	27	55
237	140	78	NT	NT
3503	5004	2770	NT	NT
41	24	22	27	26
15	23	21	12	26
4576	30	42	NT	NT
192	8100	3038	NT	NT

although the response patterns of HA1.9 and HA2.43 appeared to "split" the DR1/Dw1 antigenic complex since no DR1$^-$/Dw1$^-$ APC was able to present antigen to them. From these data we conclude that four TLCs, each recognizing one of three discrete HA determinants, were also restricted by at least three distinct interaction elements.

MoAb inhibition of TLC proliferation. To further define the class II molecules acting as restriction elements, monoclonal antibodies against specific human class II epitopes were used to block TLC proliferation (Fig. 1). The clones HA1.4 and HA1.7 that presumably recognized the same HA determinant were also blocked identically by different MoAbs. While the anti-DR SG157 (Goyert et al. 1982a) produced little relative attenuation of the response by HA1.4 and HA1.7, the response of both

Table 4. Presentation of influenza virus by HTC lymphoblastoid B-cell lines (cpm)

LCL	HLA-D specificity	HA1.4+Ag	HA1.4−Ag	HA1.7+Ag	HA1.7−Ag
AR	Dw1	6116	1172	5700	828
AP	Dw2	680	696	684	502
AQ	Dw3	248	206	350	330
7618	Dw4	6727	918	5559	1030
7336	Dw5	369	328	321	294
7617	Dw6	723	668	747	640
7380	Dw7	759	846	703	778
AM	Dw8	1089	994	1051	950
7339	Dw9	521	474	562	592
7337	Dw10	894	988	937	986
Med	–	9	–	18	–
TCGF	–	1059	–	2205	–

TLCs at 10^4/well were added to cultures containing 2.5×10^4 LCL cells (irradiated with 60 Gy) and A/Texas at 10 HAU/ml or medium. Note that LCL 7337 is DR4-positive.

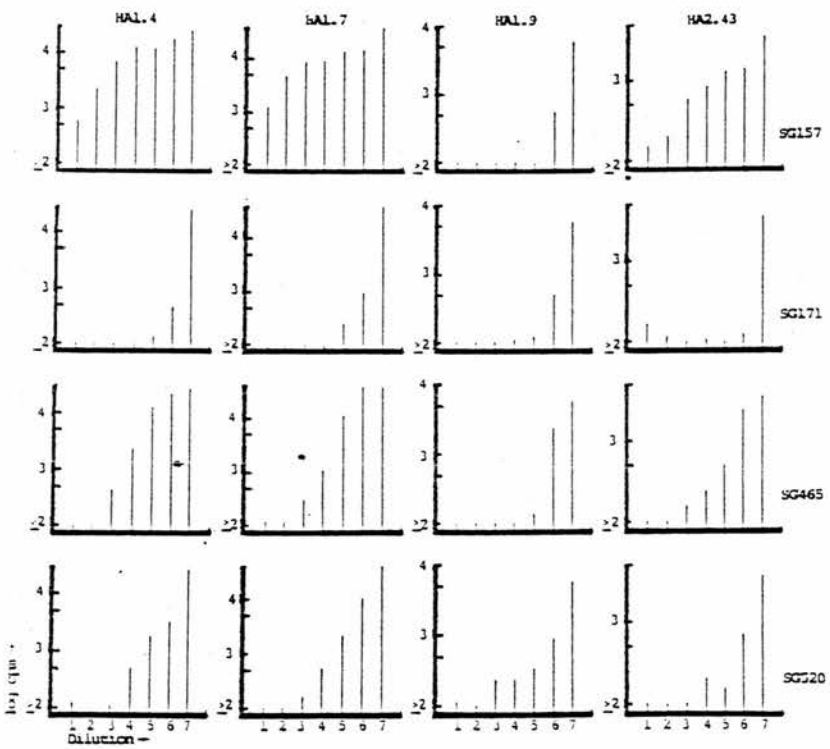

Fig. 1. The blocking effects of monoclonal antibodies SG157, SG171, SG465, and SG520 on clones HA1.4, HA1.7, HA1.9, and HA2.43. Ten thousand TLC cells were incubated with 5×10^4 irradiated (30Gy) autologous PBLs (donor 144) in the presence of MoAb for 72 h. The dilutions 1–6 correspond to serial 1:3 dilutions; dilution 7 corresponds to the untreated medium control. The starting concentrations of MoAb ranged from approximately 10–50 μg/ml.

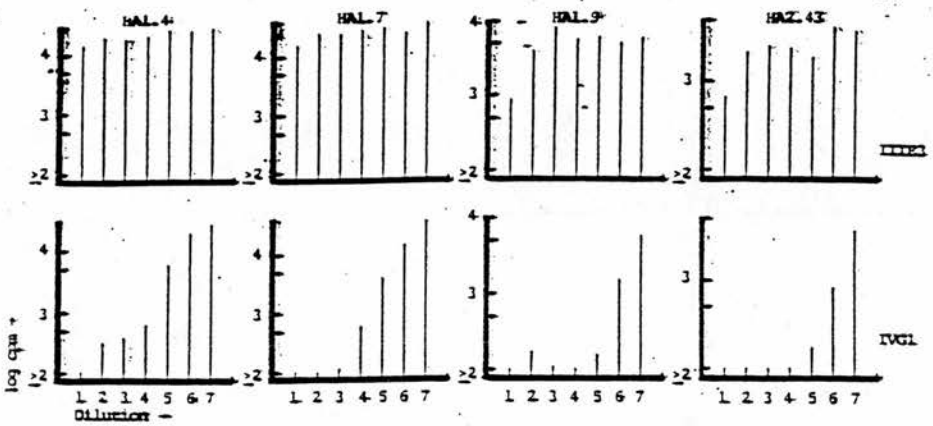

Fig. 2. The blocking effects of MoAbs IIIE3 and IVG1 on HA1.4, HA1.7, and HA2.43. IIIE3 and IVG1 recognize different determinants of the same DR-related class II molecules on some cell lines. See legend to Figure 1 for methodology. The presenting cell in this figure is derived from donor 144 (autologous); note different effect of these MoAbs on different presenting cell (donor 22; cf. Fig. 3).

clones was markedly inhibited by the MoAbs SG171 (Goyert et al. 1982b), SG465 and SG520 (Goyert et al. 1983) which recognize a second subset of human class II molecules (DS/DC). This result was in contrast to the restriction patterns for these clones which could not be correlated with any known *MB/MT/DC* allele. All of the MoAbs, SG157, SG171, SG465, and SG520, markedly inhibited responses to A/Texas by the TLCs HA1.9 and HA2.43 in the presence of autologous APCs.

The MoAbs IIIE3 and IVG1 may recognize different epitopes on the same set of HLA-DR molecules (Hurley and Nunez 1982). While IIIE3 had virtually no effect on HA1.4 and HA1.7 (Fig. 2), the responses of clones HA1.9 and HA2.43 were partially abrogated by this MoAb at the highest concentration (81% and 85% inhibition, respectively). IVG1 profoundly reduced proliferation by HA1.4, HA1.7, HA1.9, and HA2.43 to background levels. Interestingly, comparison of these MoAbs ability to block HA1.4 and HA1.7 on APCs from donor 22 revealed that while IIIE3 behaved as before, IVG1 was no longer capable of completely blocking responses by the TLCs (Fig. 3).

The Q2/70 MoAb (Quaranta et al. 1981) completely ablated responsiveness by the HA1.9 TLC while not significantly inhibiting the responses of HA1.4, HA1.7 and HA2.43 on autologous APCs (Fig. 4). The MoAb Q5/6, on the other hand, inhibited all the HA-specific TLCs with equal effectiveness.

The results from these experiments support the argument that the four TLCs may be restricted by three distinct restriction elements. Significantly, these data further indicate that HA1.4 and HA1.7 could recognize restriction elements that may correspond to epitopes of a DS/DC/MB/MT-associated class II molecule.

Discussion

Since the initial description of genetic restriction (Shevach and Rosenthal 1973, Rosenthal and Shevach 1973), much effort has been expended towards determining

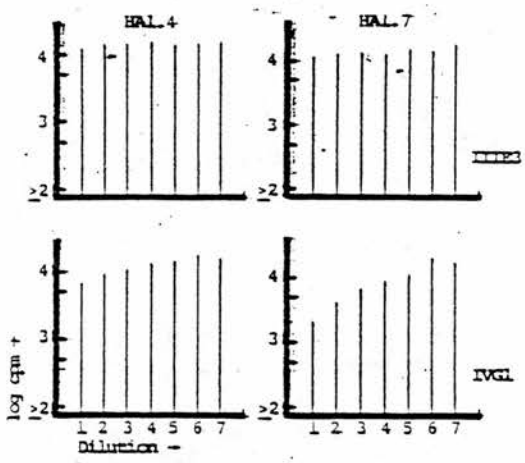

Fig. 3. Loss of blocking effect by IVG1 on HA1.4 and HA1.7 when allogeneic (DR4⁻; Dw4⁻) presenting cells from donor 22 were used. See legend to Figure 1 for methodology. Compare with appropriate panels in Figure 2.

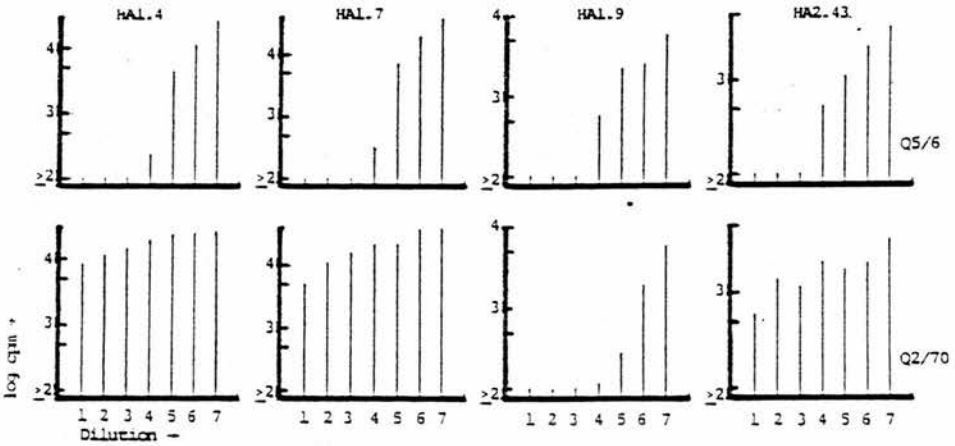

Fig. 4. Differential blocking of HA1.9 proliferation by Q2/70. See legend to Figure 1 for methodology. Note that HA1.9 is restricted by a different D-region epitope as compared with HA1.4, HA1.7 and HA2.43 in panel studies.

the mechanism of *Ir* gene phenomena. The early studies tended to indicate that nonresponder presenting cells were incapable of presenting relevant antigenic determinants to primed syngeneic T cells. However, it was later shown that bone marrow cells from responder animals, when allowed to differentiate in the presence of nonresponder thymuses, acquired the nonresponder phenotype (Zinkernagel et al. 1978 Miller et al. 1979). Furthermore, relatively recent studies indicate that *Ir* gene phenomena may be due to missing clones or "T-cell holes" (Schwartz 1978, Ishii et al. 1983). By eliminating alloreactive T cells using BUdR and light, Ishii and co-workers (1981) were able to show that nonresponder APCs were able to present antigen to responder primed T cells. These results have been confirmed by several

laboratories (Clark and Shevach 1982, Thomas and Hoffman 1982) including that of Ford and co-workers (1982) who demonstrated that the genetic restriction of human T cells is imposed by the HLA-DR type of the APCs used in primary culture. Therefore, it seems that Ia antigens play an important role in selecting the T-cell repertoire during ontogeny as well as during postthymic maturation, a concept which is further extended by the results of Sredni and Schwartz (1980) demonstrating T-cell clones specific for DNP-ovalbumin that "cross-react" with allogeneic Ia antigens. Although these results are consistent with a "T-cell hole" theory, it remains possible that within the constraints imposed by the available T-cell repertoire, determinant selection still occurs and may be controlled by the Ia antigens of the APC, a concession made also by Ishii and co-workers (1983). Further evidence for determinant selection has been provided by Werdelin (1982), Heber-Katz and co-workers (1982), Rock and Benacerraf (1983), and Matis and co-workers (1983). The common denominator in all of these latter experiments is that there is a demonstrable, albeit subtle, interaction directly between class II molecules and antigen. Therefore, determinant selection may play a viable role in the selection of responsive T-cell clones subsequent to thymic differentiation.

In this context we have demonstrated that monoclonal human T cells which recognize distinct antigenic determinants on the same HA molecule are controlled (restricted) by different *HLA-D* region epitopes. The details regarding the fine specificity of these clones have been presented elsewhere (Lamb et al. 1982c) and are only summarized herein. Clearly, HA1.9 and HA2.43 recognize different antigenic determinants from each other as well as HA1.4 and HA1.7, although it has not been possible to determine whether HA1.4 and HA1.7 do in fact recognize the same determinant on the HA molecule. In any event, the determinant(s) recognized by HA1.4 and HA1.7 is quite different from that recognized by the other two HA-specific TLCs and the restriction patterns for the four clones suggested that different genes may be controlling responses depending on the antigenic determinant recognized. If HA1.4 and HA1.7 recognize the same determinant then identical restriction patterns for these clones would be expected and are in fact what was observed. Such an expectation would also hold if HA1.4 and HA1.7 were derived from the same parental cell in the original priming culture.

It was somewhat surprising that no clear correlation was found between the restriction patterns of the clones and any one *D*-region specificity such as DR, D, SB, DC, etc. To investigate this further, we attempted to block TLC activation with MoAbs against various class II molecules. We thought that if a MoAb recognized a DC-framework epitope, for example, and preferentially interfered with clonal recognition, we could then infer something about the subclasses of Ia-like antigens controlling human immune responses. This is in fact what occurred although the conclusions must be qualified by the reservation that anti-class II MoAbs are of heterogeneous avidities, "cross-reactive" in the extreme and capable of exerting effects at the level of other interaction structures (e.g., T4) or the TLC itself. Resolution of these problems awaits a more suitable experimental system in which monoclonal antibodies against human allotopic sites are available. Also becoming increasingly clear is the fact that the framework epitopes recognized by most currently available MoAbs are represented on multiple class II molecules (S. M. Goyert and J. Silver, personal communication). Nevertheless, if the clones, HA1.4

and HA1.7, recognize the same antigenic determinant and are restricted by the same class II epitope, the patterns of inhibition with anti-class II MoAbs should be identical. Since identical blocking patterns were observed our results would therefore support this interpretation with the caveat that the determinant structure of peptide 20 needs further clarification to ascertain whether HA1.4 and HA1.7 recognize different determinants within the p20 fragment. That multiple recognition sites can be found in the p20 region of the HA1 molecule is borne out by the observation that HA2.43 recognizes both p20 and A/Japan/305 (Lamb et al. 1982c) while HA1.4 and HA1.7 recognized only p20. Of interest is the fact that responses to p20 presented by APCs from donor 22 were not blocked by the MoAb IVG1 while responses in the presence of autologous APCs were blocked. These results suggest that either the MoAb binds to different sites on different class II molecules or that different sites are recognized as restriction elements depending on the APC/TLC interaction. In this regard Heber-Katz and her colleagues have shown that single amino acid differences in the antigen can be crucial for T-cell recognition of antigen (Heber-Katz et al. 1982, Matis et al. 1983). Our results would be consistent with subtle interactions taking place at the level of the T-cell receptor and the APC class II molecule. However, whether the same class II epitope acts to restrict responses to a given antigenic determinant still remains to be resolved. This question awaits resolution within a single individual as well as the whole population.

Therefore our data would be consistent with a determinant selection type model if it is first recognized that the clones derived have already undergone thymic maturation, obviously in the presence of self class II antigens. Thus, an antigen or peptide fragment in association with different class II molecules would be expected to be recognized by different clones of T cells, each recognizing that antigen in a slightly altered context. Unlike other experiments with polyclonal T cells, these speculations need not be tempered by such concerns as those raised by Sercarz and his colleagues (reviewed by Goodman and Sercarz 1983) demonstrating that different antigenic determinants elicit specific and potentially antagonistic effects on T-cell *populations*. Indeed, T-cell clones should provide an ideal approach to such questions as they would facilitate studies of the isolated TLCs induced by different determinants which could then be further studied for interactive effects in coculture assays.

Although great strides have been made recently in the murine system, the fundamental mechanisms operating in human genetic restriction and *Ir* gene phenomena remain largely unknown. Accumulating data strongly suggests that the human genome contains many class II sequences (Mawas 1983). Since it is impossible to develop congenic human lines for the purpose of isolating and studying human class II antigenic function, TLCs should prove on ideal link between structural and functional investigations. The subtleties of T-cell recognition may seem esoteric at first glance; however, in view of evidence that so-called I-J determinants may exist as covert determinants on the I-A and/or the I-E beta-chains (Baxevanis et al. 1983, Ikezawa et al. 1983) and can act as restriction elements for suppressor T-cells, we can only assume similar mechanisms will be found to operate in man. Should this prove out in the laboratory, the clinical ramifications for MHC-linked diseases are obvious and thus human TLCs may be the only tool for dissecting such complex structural and functional relationships.

Acknowledgments. The authors acknowledge the expert assistance of Ms. Dorothy Sykes in preparing the manuscript. Special thanks are due to Drs. Nicole Sucui-Foca and Christine Rehowsky for providing the HTC lymphoblastoid cell lines. This effort was supported by grant AI 19655 from the NIAID.

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Received January 18, 1984

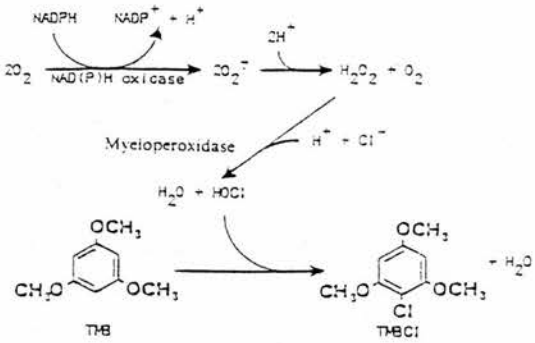

Fig. 1 Proposed scheme for HOCl production by human PMNs and its reaction with TMB.

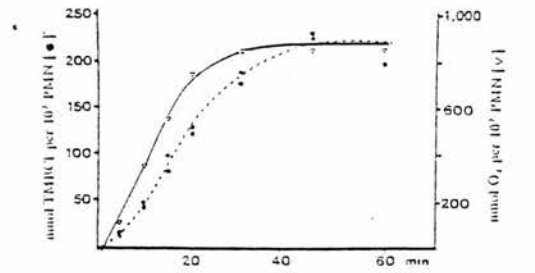

Fig. 2 Oxygen uptake and chlorination by normal human PMNs (2.5×10^6 cells per ml; prepared as previously described[17], in HEPES-buffered saline (pH 7.4, 37 °C) containing ^{14}C-TMB (1.0×10^{-4} M). The cells were stimulated with polystyrene beads (0.82 μm diameter; concentration = 1.1×10^9 beads ml^{-1}) containing adsorbed phorbol myristate acetate (50 ng per 10^{10} beads). Oxygen concentration was monitored with a Clark oxygen electrode. Chlorination was monitored by removing aliquots and measuring the concentration of TMB and TMBCl in each. Each aliquot was briefly sonicated to disrupt all cells, allowed to stand for 10 min (optional), treated with excess sodium thiosulphate and centrifuged at 13,000*g* for 2 min to pellet the polystyrene beads. The supernatant was passed through a Waters C_{18} Sep Pac precolumn followed by 6 ml of water and 6 ml of methanol. A mixture of unlabelled standards (TMB and TMBCl) was added to the methanol fraction, which was evaporated under a stream of nitrogen at 35 °C to a final volume of 200 μl. 20 μl of this solution was analysed by HPLC (Altex Ultrasphere ODS column, 4.6 × 250 mm) using isocratic elution with acetonitrile/water (45:55); UV absorption was monitored at 216 nm. The column effluent was divided using UV detection of the unlabelled standards as a guide and the ^{14}C-activity of each fraction was measured by liquid scintillation counting.

molecules (such as proteins) for the available chlorinating agents[9,13]. Indeed, chlorination of TMB (10^{-4} M) with HOCl (10^{-4} M) was 95% suppressed in the presence of 5×10^{-4} M bovine serum albumin (BSA). As this concentration of BSA is also present in the PMN buffer medium, BSA almost certainly competes with TMB for some of the PMN-generated chlorinating agents. The fact that chlorination by PMNs is not more fully suppressed by BSA suggests that much of the observed chlorination may be occurring intracellularly.

Chlorination by PMNs from a patient with chronic granulomatous disease was also measured. Because of a genetic defect, such PMNs fail to exhibit a respiratory burst[1]. Consistent with the scheme shown in Fig. 1, we could detect no (that is, ≤5 nmol per 10^7 cells) TMBCl production by these cells. Myeloperoxidase-deficient PMNs or azide-treated normal PMNs are also deficient in their ability to chlorinate TMB (data not published).

These results demonstrate the formation of strong chlorinating agents by stimulated PMNs on a similar time scale to that of expression of microbicidal activity. As the precursors of the chlorinating agents (for example, $MPO/H_2O_2/Cl^-$) appear to be localized in the phagocytic vacuole[1-5], our results support the argument[1-14] that chlorinating agents provide at least one strong microbicidal agent in the intact PMN. This new assay should prove generally useful for studying the metabolism and microbicidal function of normal and defective phagocytes.

We thank Richard Root and Julie Metcalf of Yale University for help with some of the chlorination assays. This work was supported by USPHS grants GM-20080, CA-30526 and AI-16252.

Received 6 September; accepted 6 December 1982.

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SB-restricted presentation of influenza and herpes simplex virus antigens to human T-lymphocyte clones

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The *HLA-D* region of the human major histocompatibility complex (MHC) has been shown to be homologous to the murine *I* region in terms of both structure and function. Both regions encode class II MHC molecules which restrict T-lymphocyte interactions with antigen-presenting cells. We have recently described the MHC restriction and antigen specificities of human T-lymphocyte clones directed at strain A influenza virus[1-3]. The majority of T-lymphocyte clones recognized antigen in the context of cell surface interaction products encoded by *HLA-D/DR* genes. However, a few clones recognized antigen presented by cells histoincompatible for D/DR antigens. We report here that some of these clones recognize viral antigens in association with antigens encoded by genes identical with or closely linked to the recently described secondary B-cell (*SB*) locus of the MHC[4,5]. This is the first report that SB-restricted antigen recognition may form an integral part of normal, human immune responses.

0028–0836/83/080716—03$01.00

Peripheral blood lymphocytes (PBLs) from donor 144 (HLA-A1; B8; DR1, 3; Dw1, 3; SB2, 4) were primed *in vitro* with the strain A influenza virus A/Texas/1-77/x-49. Lymphoblasts were enriched on a Percoll gradient and cloned by limiting dilution in the presence of T-cell growth factor, antigen and irradiated autologous PBLs. Details of the cloning protocol, growth, phenotyping, antigen specificities and the behaviour of subclones have been described elsewhere[1,2]. Assays for antigen presentation using histocompatible and histoincompatible PBLs required fractionation of the presenting cells into T (E^+) and non-T (E^-) fractions by rosetting with sheep erythrocytes treated with *S*-2-aminoethylisothiouronium bromide hydrobromide as described previously[1]. Proliferation, as correlated with incorporation of tritiated methylthymidine, was assessed by liquid scintillation spectroscopy. Responses were categorized as positive or negative based on the kurtosis test for response outliers as described previously[2]. In our system, T-lymphocyte clones do not respond to presenting cells in the absence of specific antigen[1,2].

The A/Texas neuraminidase-specific T-lymphocyte clone, FL1-71, was assayed for proliferation to influenza A virus presented by E^- cells from a family in which the *SB2* locus was segregating (Table 1). Within this family, FL1-71 gave significant proliferation only in the presence of $SB2^+$ presenting cells. The responses to antigen presented by $SB2^-$ cells (1077 and 1073) were 12 ± 21 and 48 ± 14 c.p.m., respectively. In contrast, cells from donor 1077 and 1073 were able to present antigen to a non-clonal cell line reactive to influenza A (CTLL A) and stimulate an alloreactive cell line (CTLL B) sensitized to a pool of allogeneic donors. (Both these primed responder cell lines were derived from donor 144.) Thus the presenting cells from donors 1077 and 1073 failed to collaborate with the T-lymphocyte clone FL1-71 although they were functionally intact. Responses to autologous (144) presenting cells and an unrelated $SB2^-$ individual are also shown in Table 1 as well as the lack of response to two $SB2^-$ individuals, 1023 and 1022. Somewhat surprisingly, CTLL A appeared to recognize antigen in conjunction with presenting cells from donor 1022 even though there exists no apparent D-region compatibility (Table 1). However, one of the T-lymphocyte clones which has been previously described[1,2] as DR1-restricted (FL1-6) also recognizes antigen presented by E^- cells from donor 1022; this result has been published elsewhere[6]. We postulate that 1022 shares an epitope with $DR1^+$ individuals that is undetectable by conventional means or, more likely, that recombination within the D region in donor 1022 has occurred. Both alternatives are under investigation. These data suggest that donors 144 and 1022 share some common presenting element, but such a finding does not alter the results suggesting that FL1-71 may be restricted by an antigen associated with SB2.

Although no recombinant families with cross-overs isolating *SB2* from *D/DR* are available, the statistical data from unrelated cell panels indicate an extremely tight association between SB2 and positive responses by FL1-71 ($P < 3 \times 10^{-4}$ by Fisher's

Table 1 The response to influenza virus segregates with SB2 on presenting cells

Antigen-presenting cell	D-region phenotype DR	D	SB	Responder cell (c.p.m.) FL1-71	CTLL A	CTLL B
1077*	3, 5	3, 5	4, 4	124	21,911	6,291
1072†	5, 4	5, DB3	2, 3	6,288	5,293	10,095
1003	3, 5	3, 5	4, 2	3,379	16,783	6,680
1074	5, 5	5, 5	4, 2	4,056	9,846	9,535
1078	3, 5	3, 5	4, 2	3,795	19,675	8,561
1073	3, 4	3, DB3	4, 3	48	18,566	5,526
144‡	1, 3	1, 3	2, 4	1,452	27,634	805
101	5, 6.3	5, 6	2, 3	8,234	8,954	6,637
1023	1, 4	1, 10	3, 4	93	25,697	4,911
1022	2, 4	12, 4	6, 7	298	6,026	10,554

PBLs from donor 144 (HLA-A1; B8; DR1, 3; Dw1, 3; SB2, 4) were suspended at 5×10^5 per ml in RPMI 1640 medium containing 10% screened, pooled human A^+ plasma, 2 mM L-glutamine, 25 mM HEPES buffer, 50 μg ml^{-1} gentamicin, 25 IU ml^{-1} Na-heparin, 1 mM Na-pyruvate and 5 haemagglutinating units (HAU) per ml of influenza A/Texas/1-77/x-49 (Lot no. 53142; Merck, Sharpe and Dohme). After 6 days at 37 °C in 5% CO_2, responding lymphoblasts were cloned by limiting dilution at 0.3 cells per well in 60-well Terasaki microtitre plates; each well contained 20 μl of medium as above and 20% T-cell growth factor (TCGF). TCGF was obtained from screened donors by stimulating irradiated PBLs with 0.1% v/v purified phytohaemagglutinin (PHA) for 48 h and collecting the supernatant. Generally, 10^7 to 10^9 cells can be obtained from each T-lymphocyte clone using described protocols[1]. For assessment of antigen-specific stimulation, $5–10 \times 10^3$ cloned cells and $5–10 \times 10^3$ E^- antigen-presenting cells were added together in triplicate to 200 μl of supplemented medium containing 5 HAU of virus per ml in 96-well, U-bottom plates. After incubation for 3 days each well was pulsed with 1 μCi of tritiated methylthymidine and incubated overnight. Cultures were collected onto glass fibre filters and the radiolabel incorporation was measured by liquid scintillation spectroscopy. The data are expressed as the mean of triplicate cultures. The standard error of each triplicate averaged less than 20% of the mean and is therefore not presented. Additionally, as no alloreactivity has ever been observed with this system, controls of the responding T-cell plus presenting cells without antigen have been omitted. The cell line CTLL A was derived from donor 144 primed *in vitro* to influenza as above and expanded in TCGF to provide a positive control. The cell line CTLL B was also derived using PBLs from donor 144 except that the cells were primed against a pool of irradiated allogeneic PBLs from five different donors before expansion in TCGF. Detailed descriptions of the cellular typing protocols used to detect HLA-D and SB antigens are found in refs 3 and 4.

* Father.

† Mother.

‡ Autologous control (TLC donor) and positive control for CTLL A and negative control for CTLL B.

Table 2 Contingency analysis of associations between FL1-71 responses and various D-region antigens

Specificity of presenting cells	FL1-71/antigen +/+	+/−	−/+	−/−	Fisher's exact probability	Corrected probability ($n = 19$)
DR1	2	3	5	6	0.635	NS
DR2	0	5	1	10	0.688	NS
DR3	0	5	5	6	0.106	NS
DR4	2	3	3	8	0.861	NS
DR5	2	3	2	9	0.937	NS
Dw6	1	4	2	9	0.786	NS
DR7	0	5	0	11	1.0	NS
DRw8	0	5	1	10	0.688	NS
Dw9	0	5	1	10	0.688	NS
Dw10	0	5	1	10	0.688	NS
Dw11	0	5	0	11	1.0	NS
Dw12	0	5	1	10	0.688	NS
SB1	0	5	4	7	0.181	NS
SB2	5	0	0	11	2.289×10^{-4}	4.349×10^{-3}
SB3	2	3	2	9	0.937	NS
SB4	3	2	10	1	0.214	NS
SB5	0	5	0	11	1.0	NS
SB6	0	5	1	10	0.688	NS
SB7	0	5	1	10	0.688	NS

In panel experiments, E^- antigen-presenting cells from 16 unrelated donors and the autologous control (144; HLA-A1; B8; DR1, 3; Dw1, 3; SB2, 4) were tested for their capacity to present influenza A/Texas/1-77/x-49 to the T-lymphocyte clone FL1-71. Proliferation assays were performed as described in the legend to Table 1. Representative data can also be seen in Table 1. Responses were classified as positive or negative based on a test for outliers using the kurtosis statistic as described previously[2]. The data were divided into positive and negative responses and the results were analysed in a 2×2 contingency table and the probability of association (P) between *D*-region alleles and positive responses by FL1-71 was computed using Fisher's exact test. Although only a small panel has been tested, the results with SB2 are highly significant but they do not prove identity between SB2 and the interaction product recognized by the clone. NS, not significant.

Table 3 Responses of an HSV-specific, SB-restricted T-lymphocyte clone

Donor	Phenotype DR	D	SB	Proliferative response (c.p.m.) A53 − media	A53 − HSV
1053	3, 7	3, 7	1, ?	49	55
101	5, 6.3	5, ?	2, 3	78	36
1022	4, 12	4, 2	6, 7	1,460	1,057
1199	3, ?	3, 5	1, 4	468	21,287
1003	3, 5	3, 5	2, 4	90	19,837
144*	1, 3	1, 3	2, 4	47	4,616
1011	1, ?	1, 4	2, 4	24	12,915
1023	1, 4	1, 10	3, 4	51	8,663
1005	2, 7	2, 7	4, ?	21	14,204
506	4, 6.1	4, ?	4, ?	159	18,581
694	1, 5	ND	4, ?	151	19,385
1009	2, 7	2, 7	4, ?	47	19,120
9999	ND	ND	4, ?	191	15,041

PBLs from donor 144 (HLA-A1; B8; DR1, 3; Dw1, 3; SB2, 4) were primed to UV-inactivated lysates of HSV-infected VERO cells and cloned in a protocol as described in Table 1 legend. For assay, 10^4 of the cloned cells were added to 10^4 irradiated E⁻ antigen-presenting cells from 13 unrelated donors (including the autologous control, 144) who were previously typed for HLA and SB antigens. Culture conditions and assay procedures were identical to those described for the influenza system. Clone A53 gave positive responses only in the presence of antigen and E⁻ cells bearing SB4. The presenting cell, 1022, appeared to be stimulatory even in the absence of antigen and was therefore considered negative relative to other cells giving specific responses.

ND, not done.

* Autologous control (TLC donor).

exact test; Table 2). Even if this value is corrected by the number of antigenic specificities typed for ($n = 19$), the result remains highly significant.

In another system, T-lymphocyte clones were generated from the same donor (144) against herpes simplex virus (HSV) and one clone was identified (A53) which appeared restricted by SB4 (Table 3); essentially background level responses were obtained when SB4⁻ antigen-presenting cells were used to present HSV to A53. Such data from two different antigen systems confirm the generalization that the *SB* region may indeed encode functional interaction products. However, even though such data reveal a high degree of association they do not, at this stage, prove identity between SB2 and the interaction product recognized by the clone.

At present, there is no apparent correlation between restriction specificities and the antigen specificity or function of the T-lymphocyte clones. However, further experiments are required to define the complete functional capabilities of *SB*-region products. Although a clear functional dichotomy exists for molecules encoded by *I-A* and *I-E* in the mouse, this is not the case for *SB* and *DR* which both appear to code for *I-E*-like α and β chains[7]. Other data from our laboratory suggest that both *D/DR* and *SB* may comprise gene clusters encoding multiple cell-surface, class II molecules[7,8]. Furthermore, it is not yet known whether such products can associate on the cell surface to form functional interaction products or complex alloantigens as has been described for the $I\text{-}A^d$ and $I\text{-}B^w$ chains[9]. We are now using T-lymphocyte clones to dissect the human MHC to investigate the role of different class II alloantigens in controlling immune responses to defined antigenic determinants. This approach is based on the hypothesis that multiple *D*-region products, probably encoded by 'immune response' genes, interact to control the response of any given T cell to particular antigenic epitopes.

We thank Mrs E. Ketterer for technical assistance and acknowledge that the research reported here has been supported under the Office of Naval Research Contracts N00014-77-C-0747, 0748 and N00014-80-K-0909 with the funds provided by the Naval Medical Research and Development Command.

Received 29 June; accepted 20 December 1982.

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An inherited polymorphism in the human apolipoprotein A-I gene locus related to the development of atherosclerosis

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Epidemiological studies have identified elevated low density lipoprotein (LDL)[1–3] and diminished high density lipoprotein (HDL) cholesterol levels[3,4] as risk factors for coronary artery disease. The major protein component of HDL is apoprotein A-I (apo A-I), a polypeptide of 243 amino acids of known primary amino acid sequence[5]. This apoprotein serves as a cofactor for the plasma lecithin–cholesterol acyltransferase (LCAT) enzyme responsible for the formation of most cholesteryl esters in plasma, and also promotes cholesterol efflux from cells[5,7]. The primary translation product of apo A-I contains both a pre and a pro segment[8], and post-translational processing of apo A-I may be involved in the formation of the functional plasma apo A-I isoproteins[9,10]. Defective apo A-I processing may be the underlying problem in Tangier disease[11], in which patients have low plasma HDL and apo A-I levels despite normal apo A-I synthesis[12,13]. Patients have been reported with conditions distinct from Tangier disease in whom severe deficiency or absence of apo A-I has been associated with very low HDL levels and severe coronary artery disease[14,15]. We have now examined the apo A-I gene in two such patients and their first degree relatives. These patients have been reported to have skin and tendon xanthomas, corneal clouding and severe premature coronary atherosclerosis associated with very low HDL levels and deficiencies of two apoproteins, apo A-I and apo C-III[15]. We show that both probands are homozygous for a defect in the apo A-I gene locus.

We have previously reported the isolation of apo A-I double-stranded (ds) cDNA clones from an adult human liver cDNA library[16]. One of these clones (pAI-113) carries an insert having a DNA sequence corresponding to the 3'-untranslated region and extending in the 5' direction to the codon representing the 94th amino acid of apo A-I. This clone was used to prepare the apo A-I probe for the experiments described here. Figure 1 shows the hybridization patterns obtained by restriction digestions of chromosomal DNA prepared from a normal individual and from one of the apo A-I-deficient patients after blotting[17] and hybridization with the apo A-I probe. In the normal individual the probe hybridized to a unique 13-kilobase (kb) *Eco*RI fragment, whereas *Eco*RI-digested DNA from the patient hybridized to a unique 6.5-kb band. To examine further the molecular basis of this difference, we performed genomic blot analysis of normal individuals and both probands showing apo A-I deficiency using various combinations of restriction enzymes (Fig. 1). The sizes of the DNA fragments hybridizing to the apo A-I probe are shown in Fig. 1 and have been compiled in Table 1. As can be seen, *Eco*RI and *Pst*I either singly or in combination with other enzymes produce fragments of different size when DNA from normal individuals and apo A-I-deficient

0028–0836/83/080718—03$01.00

Functional expression of *HLA-DP* genes transfected into mouse fibroblasts

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The HLA class II antigens are a highly polymorphic family of dimeric cell-surface glycoproteins, expressed predominantly on the surface of immunocompetent cells. They are intimately involved with the induction of the T-cell response to extrinsic antigen[1–4] and are important predisposing factors for a wide spectrum of autoimmune diseases[5]. We describe here the expression of a class II product from the *HLA-DP* (new WHO nomenclature, formerly *SB*) subregion after transfer of cloned genes into mouse fibroblasts. The transfected DP antigen is recognized by several HLA class II monoclonal antibodies and, though present in a mouse cell background, is able to function in the presentation of influenza antigen to cloned *DP*-restricted human T lymphocytes.

Molecular cloning studies show that the HLA class II region contains a minimum of six α-chain[6] and seven β-chain genes, which on sequence analysis can be resolved into the three subgroups—*DP* (formerly *SB*), *DQ* (formerly *DC*) and *DR*—defined by serological and cellular typing (for a review, see ref. 4). The *DP* region, originally defined by primed lymphocyte typing[7], contains two α and two β genes arranged in the order *DPβ2*, *DPα2*, *DPβ1*, *DPα1* (ref. 8). The products of this complex, closely linked and highly related gene family are normally expressed coordinately, posing problems in attributing structural features, such as antigenic epitopes, and functional properties, such as T-cell recognition, to any one antigen. The transfection and expression of cloned genes is thus a useful approach to characterizing the structure and function of each product in isolation.

Expression after gene transfer of both human and mouse major histocompatibility complex (MHC) class II genes has been reported[9–12]. In the mouse, the functional activity of the transfected molecules was also investigated and the capacity of B-cell lymphomas[10], interferon-induced macrophages[11] and L-cell fibroblasts[12] to present antigen after transfection was demonstrated. The B-cell transfectants were also shown to effect allostimulation[10] and the L-cell transfectants to act as targets for allogeneic killing[12]. The present experiments aimed to demonstrate the expression of human *HLA-DP* genes in mouse L cells and to determine their ability to activate *DP*-restricted influenza virus-specific T cells.

The cosmid clone JG8a, isolated from a library of placental DNA, contains two *DPα*-related genes and one *DPβ*-related gene[3] (Fig. 1). The genes were introduced into mouse L cells by co-precipitation with calcium phosphate followed by selection for thymidine kinase (TK). An initial analysis of TK$^-$ transfectants for expression at the messenger RNA level showed that transcription was occurring from all three genes (data not shown). On testing mass cultures of JG8a transfectants with a variety of HLA class II monoclonal antibodies, DA6.231 (anti-DR, -DQ and -DP) gave consistent, above-background staining on flow microfluorimetry analyses using the fluorescence-activated cell sorter (FACS). This antibody was then used to select for transfectants with comparatively high levels of DP expression, either by FACS sorting or screening of primary TK$^-$ transfectants. The experiments described here analyse the antibody-binding patterns and functional activity of one such selected line.

The pattern of antibody binding to the DP transfectants is illustrated by the data from FACS analysis and from an enzyme-linked immunosorbent assay (ELISA) (Fig. 2*a*, *b*). The results show positive binding of four HLA class II monoclonal antibodies, DA6.231 (anti-DR, -DQ and -DP), TU39 (anti-DR, -DQ and -DP), SG171 (anti-DR, -DQ and -DP) and B7/21 (anti-DP, originally anti-FA, see below and ref. 13), but only background binding with DA6.164 (anti-DR), L203 (anti-DR), TU22 (anti-DQ, possibly also anti-DR) and MHM4 (classified as anti-DP). The locus specificity attributed to these antibodies is based on binding studies of γ-irradiation-induced B-cell deletion mutants selected for loss of class II expression[14,15] and immunochemical analysis on two-dimensional gels[15,16]. In the case of DA6.231,

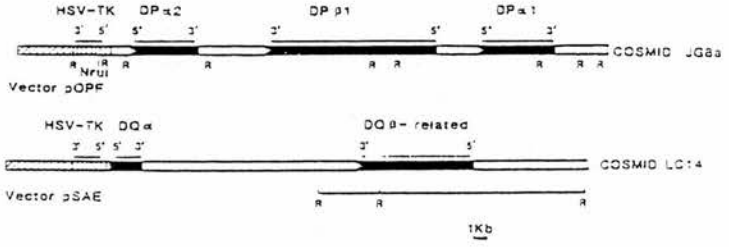

Fig. 1 Schematic maps and transfection of cosmids JG8a and LC14. Cosmid JG8a contains two DP-region α-chain genes and one β-chain gene. The restriction sites for *Eco*RI and *Nru*I are shown. Further details have been published elsewhere[3]. Cosmid LC14[26] contains an incomplete DQ_α gene and a DQ_β-related gene found on *Eco*RI fragments of 5.5 and 18 kb as shown. **Methods:** DNA was introduced into a subline of LTK$^-$ cells previously shown to give good surface expression of transfected *HLA-DR* genes[9]. The technique adopted was that of co-precipitation with calcium phosphate[27] using purified cosmid DNA and LTK$^-$ carrier DNA at a final concentration of 15 μg ml^{-1} transfection cocktail. The precipitate was added to LTK$^-$ cells, seeded at a density of 0.5×10^6 cells per 9-cm dish, and given a fresh change of Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) 4 h before transfection. 0.66 ml of transfection cocktail was added to 5.5 ml medium in each dish and after 4–10 h incubation at 37 °C, 10% CO_2, a glycerol shock was given. The cells were overlaid with 25% glycerol in DMEM for 1 min before washing and adding 10 ml DMEM with 10% FCS and 5 mM sodium butyrate[28]. After a further 12 h incubation, the medium was changed to selective medium containing 15 μg ml^{-1} hypoxanthine, 0.2 μg ml^{-1} methotrexate and 5 μg ml^{-1} thymidine. The cells were subsequently maintained in this medium with changes every 3–4 days. TK$^-$ colonies were visible after ~12 days and were either removed by trypsinization to form a mass culture or trypsinized individually using cloning rings. DNA used in the transfection experiments to produce the lines described was as follows. L8a.5: 5 μg JG8a cut with *Nru*I, 1 μg pOPF cut with *Eco*RI, 5 μg LTK$^-$ DNA; Lp0PFm: 1 μg pOPF cut with *Eco*RI, 9 μg LTK$^-$ DNA; L14m: 2 μg LC14, 8 μg LTK$^-$ DNA. The co-transfection procedure used to produce L8a.5 reduced the efficiency of TK$^-$ colony formation at least 10-fold whilst improving the levels of DP expression observed in the resulting transfectants. The *Nru*I digestion linearizes the cosmid and destroys thymidine kinase activity, while cutting at the *Eco*RI site at the 5′ end of the *tk* gene truncates important regulatory sequences and reduces transcription levels. Use of this *Eco*RI fragment has been described in constructs to promote high copy number integration[29]. The line L8a.5 was derived from a single TK$^-$ colony and selected for further study on the basis of comparatively high levels of DP expression. Southern blot analyses demonstrated the high copy number of integrated sequences found (data not shown). The lines Lp0PFm and L14m are mass cultures derived from ~100 colonies and are used as controls in subsequent experiments. Before experimental analysis, the L cells were incubated for 16–24 h in medium containing 5 mM sodium butyrate, a procedure found to enhance levels of DP expression.

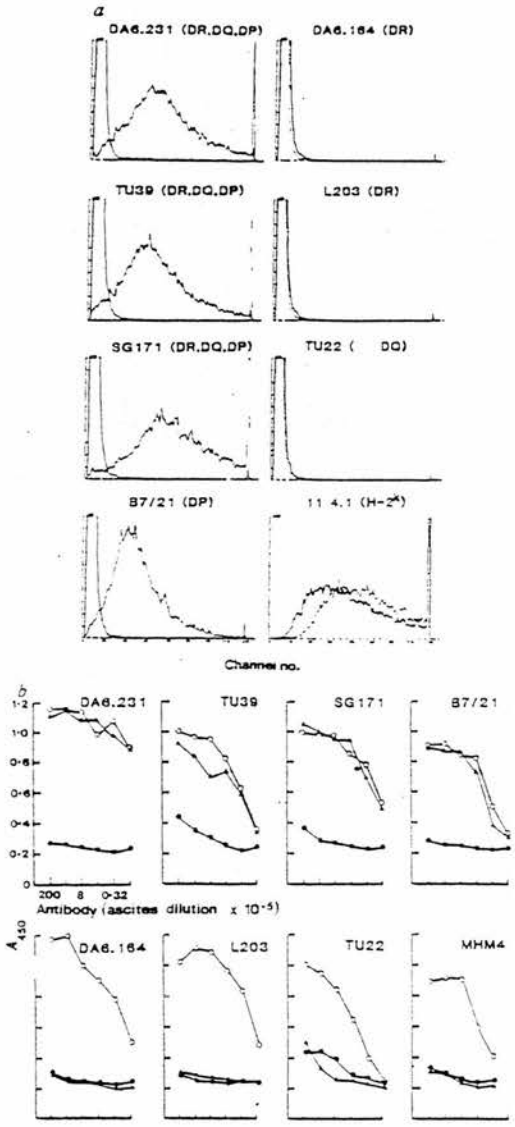

Fig. 2 Surface expression of DP antigen by transfected mouse L cells. *a*, Flow microfluorimetry analyses of the transfectants L8a.5 (thin lines) and L14m (thick lines) using a panel of monoclonal antibodies to HLA class II determinants and an anti-H-2^k class I antibody. Fluorescence intensity is plotted as a linear function of cell number and 10,000 cells were analysed for each histogram. *b*, ELISA assay for binding of HLA class II monoclonal antibodies to the EBV-transformed lymphoblastoid cell line LNAT (○) and L-cell transfectants Lp0PFm (●) and L8a-5 (▲).

Methods: *a*, 1×10^6 cells were washed with cold phosphate-buffered saline (PBS) containing 5% fetal calf serum (FCS) and incubated with 100 μl of antibody diluted in PBS + 5% FCS for 30 min on ice. All HLA class II antibodies were used in the form of ascites fluid diluted 1 in 200; 11 4.1 was used as a hybridoma supernatant diluted 1 in 2. Cells were washed three times in cold PBS + 5% FCS and incubated for a further 30 min on ice with a 1 in 10 dilution of fluorescinated IgG fraction rabbit anti-mouse IgG (Cappel Laboratories). After two final washes the cells were resuspended in cold PBS and analysed on a FACS-1 (Becton & Dickinson). *b*, Cells were washed three times in PBS and added to 96-well plates pretreated for 1 h with 0.1 mg ml^{-1} poly-L-lysine in PBS (10^5 cells in 50 μl PBS per well). The cells were spun onto the base of the wells and then fixed by gently adding 200 μl per well 0.025% glutaraldehyde in PBS and standing at room temperature for 20 min. After washing, the plates were stored at 4 °C in a solution of 200 μg ml^{-1} gelatin, 0.02% NaN_3 in PBS. Before adding antibody, the cells were treated with 0.1% phenylhydrazine in PBS for 1 h and then washed in PBS (3×) and once in PBS + 0.2% Tween 20. Antibody ascites, diluted at 1 in 500 in PBS + 5% FCS and at subsequent five-fold serial dilutions, were added to the cells at 50 μl per well in triplicate and the plates were incubated at room temperature for 2 h. The cells were washed (as above) and then incubated for 30 min with 25 μl per well 5% goat anti-mouse immunoglobulin (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service) in 200 mM Tris-HCl *p*H 7.6 with 10% normal goat serum (NGS). After further washes the plates were incubated for 30 min with peroxidase–antiperoxidase immune complexes (PAP) in Tris-HCl 7.6 + 10% NGS. After final washing, substrate (1 mg ml^{-1} *o*-phenylenediamine, 0.05% H_2O_2 in substrate buffer, 156 mM Na_2HPO_4, 27 mM citric acid *p*H 6.0) was added (100 μl per well) and the plates were incubated in darkness for 30 min. Absorbance at 450 nm was read on the Titer Multiscan.

Results are expressed as the mean of triplicate wells.

TU39, SG171, TU22, L203 and DA6.164, the binding results for the DP transfectants strengthen the argument for these locus assignments. Positive binding of B7/21 supports the view that this antibody detects DP-region molecules[17], previously termed FA[12]. Conversely, the lack of binding by the MHM4 antibody questions its specificity for DP, although it may well recognize the *DPβ2* product or a polymorphic *DPβ1* product, not expressed by the transfectants. These results emphasize the value of such transfectant lines in elucidating the complex cross-reactions of HLA class II antibodies. Accurate quantitation of the level of surface antigen expression relative to that seen on B-cell lines is difficult because of these cross-reactions. However, comparison of mean fluorescence levels observed on binding B7/21 to the B-cell line LNAT suggests that surface expression is approximately fivefold lower on the DP transfectants.

The presence of the DP antigen was confirmed biochemically by immunoprecipitation using the monoclonal antibodies SG171 and B7/21 coupled with two-dimensional non-equilibrium *p*H gradient electrophoresis/sodium dodecyl sulphate polyacrylamide gel electrophoresis (NEPHGE/SDS-PAGE) analysis. Figure 3 shows the two-dimensional pattern of ^{125}I-labelled polypeptides derived from microsomes of the mouse L cells (Fig. 3*a*) and the L cells transfected with cosmid clone JG8a (Fig. 3*b*). In addition to the nonspecific polypeptides found in both cells, the monoclonal antibodies precipitated a single putative α-chain of relative molecular mass (M_r) 30,000–32,000 and at least two possible β subunits of 29,000–32,000 from the DP-transfected cells. The identification of these spots is based on the isoelectric points and M_r positions previously found for HLA-D region antigens[17,18]. The labelled α-chain spot suggests

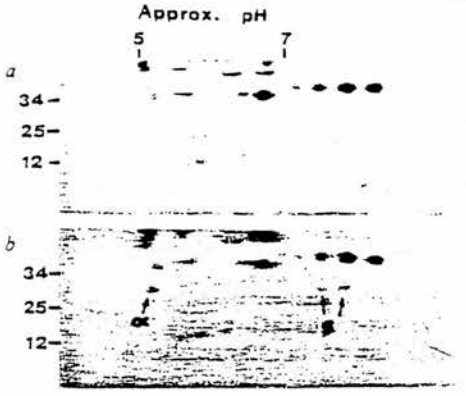

Fig. 3 Two-dimensional NEPHGE/SDS-PAGE analysis of immunoprecipitates using a combination of monoclonal antibodies SG171 and B7/21 against mouse L cells (*a*) and mouse L cells transfected with cosmid clone JG8a (*b*). Numbers on the left-hand side represent size markers ($Mr \times 10^{-3}$).

Methods: Microsomes were prepared by shearing the cells in a Stansted cell disrupter, centrifuging at 1,500 r.p.m. for 5 min to remove nuclear debris and finally at 18,000 r.p.m. for 30 min to pellet the membrane vesicles. The resuspended pellet was labelled with ^{125}I by lactoperoxidase-catalysed iodination[22], washed and then solubilized in lysis buffer (1% (w/v) Nonidet P-40 in 10 mM Tris-HCl buffer *p*H 8.2 containing 150 mM NaCl, 1 mM EDTA and 1 mM phenylmethylsulphonyl fluoride). A glycoprotein fraction was prepared by binding to *Lens culinaris* lectin and eluting with 10% α-methyl-D-mannoside. The lysate (5×10^7 cell equivalents) was incubated overnight at 4 °C with 5 μl of ascitic fluid of SG171 and B7/21 before precipitating the immune complexes with 20 μl of 10% *Staphylococcus aureus* Cowan I strain (SaC1). Immunoprecipitates were washed twice in lysis buffer containing 500 mM NaCl and once in lysis buffer alone. Immune complexes were eluted from SaC1 pellets by incubating in isoelectric focusing sample buffer (9.2 M urea, 2% Nonidet P-40, 5% ampholines *p*H range 3.5–10 (LKB) at 50 °C for 30 min. Two-dimensional NEPHGE/SDS-PAGE analysis was done as described previously[20,21].

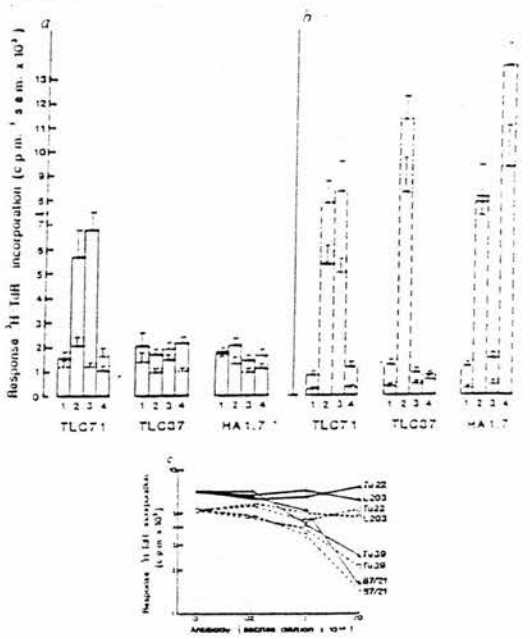

Fig. 4 Antigen presentation assay. The proliferation response of T-lymphocyte clones TLC71 (neuraminidase specific, DP-restricted), TLC37 (matrix protein specific, DR-restricted) and HA1.7 (peptide 20 specific, DQ-restricted) in the absence of antigen (column 1), or with intact virus (A/Texas/1/77 × 49 at 5 haemagglutinating units ml^{-1}, column 2), or neuraminidase (N_2 of Papua New Guinea/1/75 at 10^{-3} v/v, column 3), or p20 (1.0 μg ml^{-1}, column 4). Presenting cells: *a*, DP L-cell transfectants L8a.5 (——) (1.25×10^5 ml^{-1}); DQ L-cell transfectants L14m (· · · · ·) (1.25×10^5 ml^{-1}). *b*, Autologous PBL (— · — · —) (1.25×10^5 ml^{-1}); EBV-transformed autologous B-cell line LNAT (- - - - -) (0.5×10^5 ml^{-1}). *c*, Inhibition of the proliferation response by TLC71 with anti-HLA Class II antibodies. The assay for the proliferative response to intact virus was performed as before using DP transfectants L8a.5 (▲) and autologous PBL (C) as presenting cells. Antibody ascites was added at the initiation of the cultures in the dilutions shown, and left in for the duration of the experiment.

Methods: The isolation and characterization of T-lymphocyte clones TLC71, HA1.7 and TLC37 have been described in detail elsewhere[9,20,33,34]. Briefly, PBL from the donor LNAT were cultured for 6 days with specific antigen. The lymphoblasts were enriched on a discontinuous Percoll (Pharmacia) gradient and resuspended in RPMI-1640 (Gibco) containing 10% A^+ serum and 20% interleukin-2 (IL-2) and plated at one cell every third well in Microtest II trays (Falcon) with 10^4 irradiated (2,500 rad) autologous PBL and antigen. After 7 days, growing clones were transferred to 96-well microtitre trays and subsequently to 24-well trays. At each transfer the clones received fresh IL-2 and irradiated PBL together with antigen. The clones were expanded in 25-cm^2 tissue culture flasks receiving IL-2 every 3 days and irradiated histocompatible PBL and intact virus (A/Texas/1/77) every 7 days. IL-2 was prepared from 48-h supernatants of PBL (1×10^6 ml^{-1}) cultured with 0.1% purified phytohaemagglutinin (PHA-P, Difco) in complete medium containing 2.5% A^+ serum. The clones were used in proliferation assays 6–7 days after the addition of feeder cells (irradiated PBL). Cloned T cells (5×10^4 ml^{-1}) were cultured together with the antigens and presenting cells described above. The L-cell transfectants and EBV-line received 5,000 rad and PBL 3,000 rad before use. Following 60 h incubation, the cultures were pulsed for 8–16 h with 1.0 μCi ^3H thymidine (^3H-TdR, Amersham) and collected onto glass fibre filters. Proliferation as correlated with ^3H-TdR incorporation was measured by liquid scintillation spectroscopy. Results are expressed as the mean counts per min of triplicate cultures ± s.e.m.

that, contrary to the case of the DP α-chain on the surface of intact B cells[3,17], the α-chain in microsomes from the L-cell transfectants is accessible to lactoperoxidase-catalysed iodination. It is likely to correspond to the *DPα*1 gene product, as the sequence from this gene matches the sequence available for *DPα* cDNA clones and protein sequence data[3]. However, the data do not exclude concomitant expression of the *DPα*2 gene. The presence of multiple β-chains probably reflects stages of glycosylation[18].

Having established that the JG8a transfectants were expressing a DP antigen at the cell surface, the L-cell transfectants were assayed for their ability to induce proliferation of the T-lymphocyte clone TLC71 in the presence of antigen. TLC71 has been shown to proliferate in response to DPw2 class II determinants[19] and the neuraminidase (NA) glycoprotein of influenza A virus[20]. In the presence of intact influenza virus (A/Texas/1/77) or NA (N_2 of Papua New Guinea/1/75), the DP L-cell transfectant was able to induce TLC71 to proliferate (Fig. 4*a*). However, in the presence of an irrelevant antigen, peptide 20 (p20; residues 306–329 of the HA1 molecule of influenza haemagglutinin[21]) or with DQ transfectants, there was no response. Neither HA1.7 (*DQ* restricted and specific for p20) nor TLC 37 (*DR* restricted and specific for matrix protein) T-cell clones were activated by the L-cell transfectants. However, both HA1.7 and TLC 37 clones proliferated in response to specific antigen presented by autologous peripheral blood lymphocytes (PBL) or Epstein–Barr virus (EBV)-transformed B

cells (Fig. 4*b*). These results show that T-cell activation by the DP transfectants is both *HLA* dependent and antigen specific.

To confirm the specificity of the transfected DP molecule in antigen-induced activation of TLC71, the inhibitory effect of antibodies to various class II HLA molecules was tested. Figure 4*c* shows that the anti-DP antibodies TU39 and B7/21 inhibit the proliferation of TLC71 whereas TU22 and L203 have no effect. In a parallel experiment, TU22 (anti-DQ) and L203 (anti-DR) effectively inhibited the response of HA1.7 (*DQ*-restricted) and TLC37 (*DR*-restricted), respectively, to intact A/Texas in the presence of autologous PBL (data not shown). Further analysis of the polymorphic specificity of T-cell recognition can now be pursued using both transfectants and T-cell clones of different specificities.

The capacity of the DP-expressing mouse fibroblasts to activate human T cells in the presence of antigen indicates that antigen presentation is not restricted to the macrophage/monocyte or B-cell lineages. This agrees with our recent observation that thymocytes expressing class II antigens can activate human T-cell clones[22] and also with the results for the mouse class II L-cell transfectants[12]. The implication is that either secondary signals such as the secretion of interleukin-1 (IL-1) are not always essential or that cells other than classical antigen-presenting cells such as mouse fibroblasts are able to provide them. Similarly, the postulated interactions between the T-cell surface antigens such as T4 (refs 23, 24) and LFA-1 (ref. 25) and the antigen-presenting cell are either not obligatory or are directed against targets present on the surface of the L-cell transfectants. Requirements for T-cell activation may well be more stringent for primary rather than secondary stimulation and may also vary with the particular antigen and T-cell clone involved. However, in the system described, it is clear that antigen-dependent activation can occur in the absence of any human cell-surface molecule on the presenting cell, other than the *HLA* restriction element recognized by the T-cell receptor.

We thank Dr C. du Preval for the gift of Ltk$^-$ cells, Drs K. Guy and V. Van Heyningen for DA6.231 and DA6.164, Dr L. Trowbridge for B7/21, Dr A. Ziegler for TU39 and TU22, Dr S. Goyert for SG171, Dr A. McMichael for MHM4, Dr L. Lampson for L203, Dr A. Rosenthal (Merck Sharp and Dohme) for A/Texas/1/77, Dr R. Webster for influenza virus neuraminidase, Dr R. Lerner for peptide 20, Dr M. Contreras and her associates at the National Blood Transfusion Centre, Edgware for blood and serum, Grace Lam and Laura Davis for their assistance in operation of the FACS, Margaret Hollis and Audrey Symons for preparation of the manuscript, and Professor N. A. Mitchison and Professor Martin Raff for helpful discussion and comments.

Received 30 July; accepted 3 October 1984.

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International Immunology, Vol. 10, No. 2, pp. 211–222

Six unrelated HLA-DR-matched adults recognize identical CD4+ T cell epitopes from influenza A haemagglutinin that are not simply peptides with high HLA-DR binding affinities

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Keywords: CD4+ T cell recognition, hemagglutinin, immunodominance, immunogenetics, influenza A virus

Abstract

Influenza A/Beijing/32/92 (H3N2) haemagglutinin (HA)-specific short-term CD4+ T cell lines were generated from six unrelated HLA-DR0701, 1501 positive adults (aged 27–60 years) 3 months following administration of an influenza subunit vaccine containing HA A/Beijing/32/92. Epitope recognition was examined using 118 HA A/Beijing/32/92-specific 16mer peptides which overlapped by 11 residues and which spanned the entire molecule. Following influenza vaccination the donors recognized identical HA peptides. The selected peptides represented HA regions which have been free from extensive drift mutation since the emergence of human H3N2 influenza A strains. Using DAP DR7.0701 cells (a murine cell line expressing HLA-DR0701) as antigen-presenting cells the majority of CD4+ T cell responses were shown to be HLA-DR0701 restricted. The relationship between HA peptide recognition and relative strength of HA peptide–HLA-DR0701 binding was then explored in a competition assay with biotinylated CLIP peptide. Although peptides representing dominant HA epitopes bound to DR0701, the relationship between relative strength of binding and immunodominance was complex, and many strongly binding peptides, particularly those with glycosylation sites and showing inter-strain variation, were not recognized. These results illustrate the control HLA class II exerts over CD4+ T cell HA epitope selection in unrelated adult humans. Immunodominance appears not to be directly related to the relative strength of HA peptide–HLA class II binding, and thus reflects complex interactions between antigen processing, intracellular competition for HLA binding, TCR repertoires and repeated exposure to different strains of influenza A viruses.

Introduction

CD4+ T cells are important in defence against influenza A virus infection: as well as controlling neutralizing antibody production (1), they amplify cytotoxic T lymphocyte responses (2), and may participate more directly in viral clearance by the secretion of IFN-γ and other cytokines (3). Previously we have examined human CD4+ T cell recognition of influenza A/Beijing/32/92 (H3N2) haemagglutinin (HA) following natural infection with A/Beijing/32/92-like strains and demonstrated that in adults CD4+ T cells principally focus on HA regions which have not been subject to frequent drift mutation (conserved regions) (4). We also found a similar pattern of CD4+ T cell recognition of HA following influenza subunit vaccination (5). These studies included pairs of unrelated donors who expressed identical HLA-DR alleles and each pair responded

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Transmitting editor: A. McMichael

Received 11 July 1997, *accepted* 4 November 1997

to identical HA regions, with only minor variations in the magnitude of their responses in spite of differences in their age and exposure to influenza A viruses.

In order to extend our initial observations and to explore in detail epitope selection by HLA class II, we have now examined the HA-specific repertoires of six unrelated adults, aged 27–60 years, selected because they expressed identical HLA-DR alleles (HLA DRB1*0701, *1501). Five of the six donors also expressed the same HLA-DRW (DRB4*01, *501) and HLA-DQ alleles (DQA*0101/4, *0501 and DQB1*02, *0601/2). Short-term CD4⁺ T cell lines were selected with full length HA A/Beijing/32/92 (H3N2), prior to and 3 months following vaccination with a trivalent subunit influenza vaccine containing HA A/Beijing/32/92. Epitope recognition was examined using a panel of 118 synthetic peptides 16 amino acids in length and overlapping by 11 residues, that spanned the entire HA A/Beijing/32/92 molecule.

Because murine studies using panels of overlapping synthetic peptides spanning the sequence of staphylococcal nuclease have reported a strong association between the strength of CD4⁺ T cell peptide recognition and peptide binding affinity for MHC class II (6–8), we then employed a competition assay, using biotinylated CLIP peptide, to estimate the relative binding affinities of the HA peptides for purified HLA-DR. This allowed a preliminary analysis of the importance of peptide–HLA class II relative binding affinity in the development of a hierarchy of immunodominance for human CD4⁺ T cell responses to HA.

Methods

Subjects and vaccination

CD4⁺ T cell responses by six unrelated healthy Caucasian adult volunteers, aged between 27 and 60 years, to HA from influenza A/Beijing/32/92 (H3N2) were investigated prior to and 3–4 months following influenza subunit vaccination, although the pre-vaccination line from donor C was lost in culture. Subjects D, E and F were originally found by chance to share HLA class II types. Donors A–C were specifically chosen because they were known to have a very closely matched HLA class II type to donors D, E and F (see below). HLA-DR and DQ typing was performed using the 'phototyping' method (employing sequence specific primer PCR) (9). Donors A–C, E and F expressed identical HLA-DRB1*1501, *0701, DRB4*01, DRB5*01, DQA1*0101/4, *0501 and DQB1*02, *0601/2 alleles. Donor D differed only in that he expressed DQB1*0303 rather than DQB1*02, this is associated with the non-expression of the donor's DRB4*01 allele (10). The following HLA-DP types were obtained by sequencing: donor B, HLA-DP*0301, *0402; donor C HLA-DP*0201, *0401; and donor F, HLA-DP*0401, *0402. Donors HLA class I types, as determined by phototyping, were as follows: donor A, HLA-A3, B7,13 Cw4,6; donor B, HLA-A1,2, B62,64, Cw8,304; donor C, HLA-A30,31, B7,13, Cw6,7; donor D, HLA-A2, 28, B7,17, Cw6,7; donor E, HLA-A3,23, B7,44, Cw4,702; donor F, HLA-A2,30, B44,51.

Subjects received 0.5 ml of Influvac s.c., containing 15 μg of HA from the following influenza viruses: A/Beijing/32/92 (H3N2), A/Singapore/6/86 (H1N1) and B/Panama/45/90 (influenza B) according to the manufacturer's instructions. The vaccine was kindly donated by Dr R. Brands (Solvay Duphar, Weesp, The Netherlands).

Peptides and antigens

Highly purified full-length HA derived from influenza A/Beijing/32/92 (H3N2) was a gift from Dr R Brands. HA from influenza A/Aichi/68 (the original H3N2 human influenza strain) was the gift of Dr J. Skehel (National Institute for Medical Research, Mill Hill, London, UK) and was obtained by bromelain cleavage of the recombinant influenza A virus X-31. It contained trace contaminants of other influenza proteins (11).

A series of 16 amino acid peptides overlapping by 11 residues was derived from the nucleotide sequence of the HA1 subunit of HA A/Beijing/32/92 and the HA2 subunit of the closely related A/Hong Kong/90 virus, as no sequence information was available on the HA2 subunit of A/Beijing/32/92. Unpublished sequence information from both viruses was kindly supplied by Dr N. Cox (CDC, Atlanta, GA). The amino acid sequence and method of synthesis of the peptides has been previously described, and they showed no significant mitogenic activity on a CD4⁺ T cell line specific for *Mycobacterium tuberculosis* soluble extract (4). In the text peptides are numbered according to their N- and C-terminal residues, and peptide pools are numbered by the N-terminal residue of their N-terminal peptide and the C-terminal residue of their C-terminal peptide.

CD4⁺ T cell lines

Short-term CD4⁺ T cell lines were selected using purified full-length HA A/Beijing/32/92 as previously described (4). In brief, short-term CD4⁺ T cell lines were obtained by stimulating fresh peripheral blood mononuclear cells (PBMC) (5×10^6/well) with purified HA A/Beijing/32/92 0.05 μg/ml, in RPMI 1640 supplemented with 5% heat inactivated human AB⁺ serum (Sigma, Poole, UK), 2 mM glutamine, 100 IU/l of penicillin–streptomycin at 37° C, 5% CO_2. At 7 and 14 days T cells were re-stimulated with equal numbers of autologous irradiated (3000 rad) PBMC (freshly thawed, from liquid nitrogen storage) pre-pulsed with 0.01–0.05 μg/ml of HA A/Beijing/32/92, and cultured in complete medium at a density of 1×10^6 T cells/well. Then 10% Lymphocult (Biotest Folex, Frankfurt, Germany) as a source of IL-2 was added on days 8, 11, 15 and 18. By 21 days of culture, short-term CD4⁺ T cell lines selected as above are >90% CD3⁺, 78–88% CD4⁺ and 2–10% CD8⁺ (4).

Analysis of T cell specificities using overlapping HA peptides

At 14 and 21 days of culture autologous, irradiated (3000 rad) PBMC (4×10^4 cells/0.2 ml well) were incubated with whole HA (HA A/Beijing/32/92, 0.01–0.1 μg/ml) or HA peptide pools (five peptides at an individual concentration of 10 μg/ml) and cultured with 4×10^4 responder T cells/well. At 48 h the cells were pulsed with 1 μCi/well methyl-[^{3}H]thymidine (Amersham International, Amersham, UK) and harvested 16 h later. Proliferation was measured by liquid scintillation spectroscopy. Fine specificity of the T cells was mapped in more detail with individual HA peptides (5 μg/ml) at 14 and 21 days of culture as above.

HLA class II restriction studies

At 21 days irradiated (3000 rad) PBMC from a donor with a DRB1*0101,0701 DRB4*01 DQB1*02,05 haplotype (DR0701 donor), and a donor with a DRB1*0408,1501 DRB4*01 DRB5*01 DQB1*0301,0601/2 haplotype (DR1501 donor) were used to present HA A/Beijing/32/92 and HA peptides to developing short-term T cell lines as above.

Further HLA restriction studies were conducted on a 2 week HA/Beijing/32/92-specific CD4+ short-term T cell line derived from donor D as described above. HA peptide proliferation was examined as described above with one modification: 4×10^4 irradiated DAP.DR7.0701 cells (a murine fibroblast line which expresses DRB1*0701) were used in place of autologous PBMC as antigen-presenting cells (APC). The DAP.DR7 line was the gift of Professor R. Lechler (Royal Postgraduate Medical School, London, UK).

Purification of HLA-DR0701, 0101, 0401, 1302

HLA-DR protein was prepared from the following Epstein–Barr virus-transformed B cell lines: DR0101, LG-2 (12), DR0401, Bm 14, DR0701, LBF (13), DR1302 and WT-47 (14) as previously described (15). In brief a 10 g cell pellet was lysed in PBS containing 2% CHAPS (v/v), leupeptin (2 μg/ml), pepstatin (2 μg/ml) and 5 mM EDTA. Cell lysates were centrifuged at 100,000 *g* for 90 min at 4°C then loaded onto an L-243 affinity column. The column was washed extensively and then material eluted with 0.05 % diethylamine/150 mM NaCl/0.1% CHAPS, pH 11.5. Eluate was concentrated on a Centricon-10 column (Amicon).

Peptide binding studies

An invariant chain fragment containing the CLIP peptide (LPKPPKPVSKMRMATPLLMQ) [Ii-(97–120)] was biotinylated using biotinamidocaproate *N*-hydroxysuccinamide ester (Sigma). Binding of peptides to DR molecules was measured by their ability to inhibit the binding of the biotinylated CLIP peptide as previously described (14). Briefly, 0.1 μg of HLA-DR was incubated with biotinylated CLIP peptide (0.02 μg) with or without inhibitor peptide (10, 1 and 0.1 μg) at 37°C for 24 h at pH 5. Solutions were neutralized with Tris–HCl (pH 7.5) before transfer to wells pre-coated with L-243 antibody. Binding of biotinylated peptide was detected with ExtrAvidin–horseradish peroxidase conjugate (Sigma) and developed with *o*-phenylenediamine. The OD at 492 nm was measured using an ELISA plate reader and the concentration of each HA peptide which caused a 50% inhibition in biotinylated CLIP peptide binding calculated (IC_{50}) using standard methods.

Results

HA and HA peptide recognition

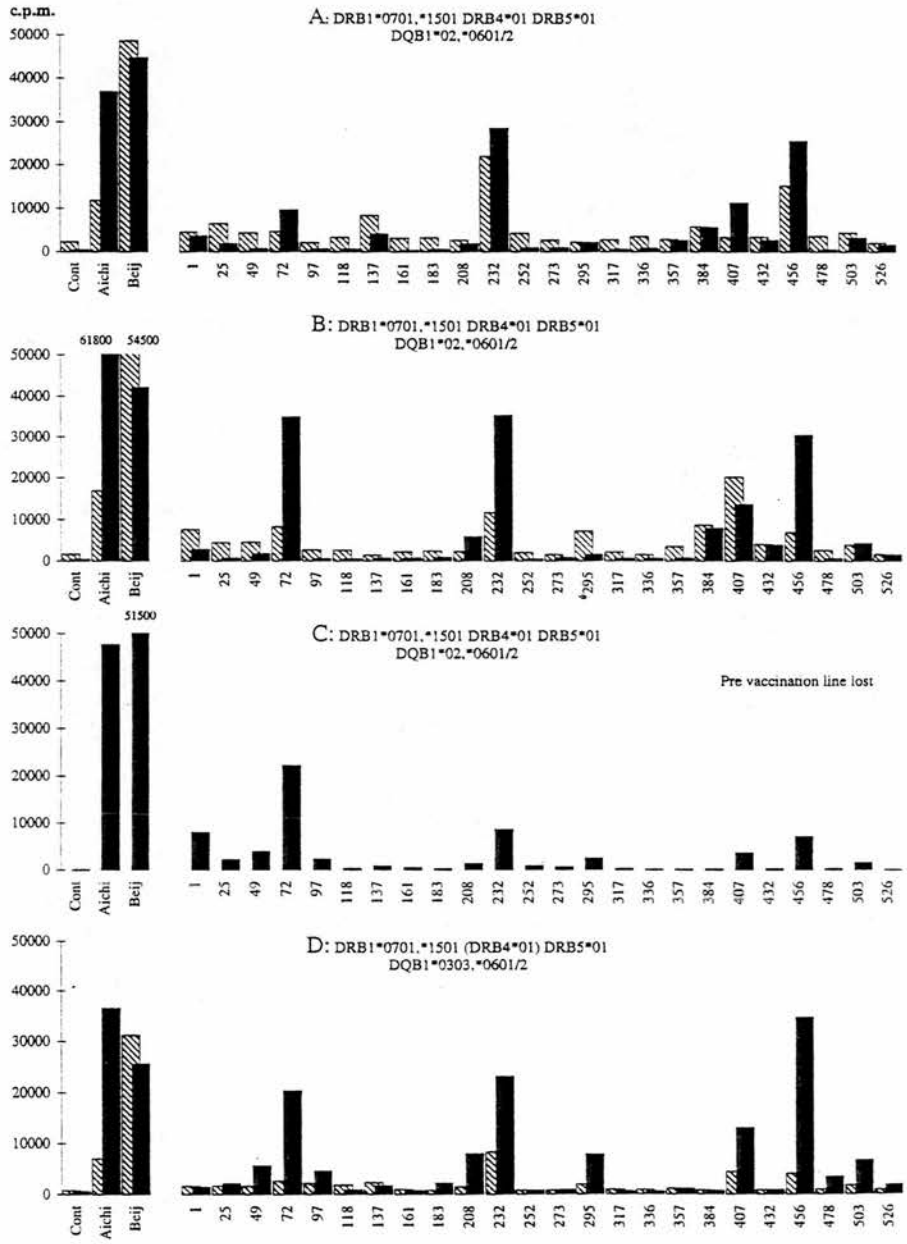
Recognition of HA and the HA peptide pools by short-term CD4+ T cell lines selected prior to HANA influenza subunit vaccination was variable in magnitude (donors A, B and D, Fig. 1, and two donors reported in ref. 5, the line from donor C was lost in culture), which probably reflects differences in their previous exposure to influenza A strains. After subunit vaccination short-term CD4+ T cell lines derived from every donor mounted strong proliferative responses to HA A/Beijing/32/92, HA A/Aichi/68, and HA peptide pools 72–107, 232–262, 407–442 and 456–488 (Fig. 1). Their minor HA responses were also similar, with five of six donors recognizing peptide pools 1–35, 49–82, 208–243, 295–328 and 503–536.

When CD4+ T cell responses to individual HA peptides within the dominant peptide pools were examined (Fig. 2), every CD4+ T cell line was found to recognize identical HA peptides or pairs of overlapping peptides, with the exception of donor E, whose responses to pools 407–442 and 456–488 could not be localized. Furthermore, where responses localized to pairs of overlapping peptides, the relative strength of responses to each individual peptide was the same. Thus the response to peptide 87–102 was stronger than the response to peptide 92–107, the response to peptide 232–247 was stronger than the response to peptide 228–243, and the responses to peptides 463–478 and 468–483 were similar to each other in magnitude (with the exception of donor E). These peptides represent regions of HA which have been free of extensive drift mutation since the emergence of the H3N2 subtype. Though the CD4+ T cell response of these donors is principally focused on conserved regions of HA, we cannot exclude the possibility that donors A, C and G have mounted minor but significant (>5 times the geometric mean background) responses to neutralizing antibody sites A, B, and/or D which are represented by peptides in pool 137–171.

HLA class II restriction of T cell responses to intact HA and HA peptides

In order to examine HLA restriction of CD4+ T cell responses to HA, PBMC from two donors with partial HLA class II matches were used as APC (Fig. 3). The 'DR0701' donor expressed HLA-DRB1*0101, DRB1*0701, DRB4*01, DQB1*05 and DQB1*02. The 'DR15' donor expressed HLA-DRB1*0408, DRB1*1501, DRB4*01, DRB5*01, DQB1*0301 and DQB1*0601/0602. When PBMC from the 'DR0701' donor were used as APC, strong T cell proliferative responses were seen to peptides 87–102, 232–247, 463–478 and 468–493, indicating that these responses were primarily restricted to either HLA-DRB1*0701, DRB4*01, DQB1*02 or HLA DP (see below). When PBMC from the 'DR1501 donor' were used as APC, T cell responses were seen to peptides 232–247 and 417–432.

CD4+ T cell recognition of peptides 87–102, 232–247, 463–478 and 468–493 is unlikely to be primarily DQB1*02 restricted because donor D, who does not express DQB1*02, had a very similar peptide recognition pattern to the other donors. These responses are also unlikely to be DRB4*01 restricted. First, both the 'DR0701' donor and the 'DR1501' donor expressed DRB4*01, yet strong responses to peptides 87–102, 232–247, 463–478 and 468–493 were only seen when PBMC from the 'DR0701' donor were used as APC; secondly, donor D, who does not express DRB4*01 (10) has a very similar HA peptide recognition pattern to the other donors; and, finally, we have previously examined five other DRB4*01 positive donors, none of whom recognized peptides 87–102 and 232–247 (4,5). CD4+ T cell recognition of peptides 87–102, 232–247, 463–478 and 468–493 is also unlikely to be primarily HLA-DP restricted because donors B, C and F express different HLA-DP alleles (HLA-DP*0301,*0402, HLA-


c.p.m.
A: DRB1*0701,*1501 DRB4*01 DRB5*01
DQB1*02,*0601/2
B: DRB1*0701,*1501 DRB4*01 DRB5*01
DQB1*02,*0601/2
61800
54500
C: DRB1*0701,*1501 DRB4*01 DRB5*01
DQB1*02,*0601/2
51500
Pre vaccination line lost
D: DRB1*0701,*1501 (DRB4*01) DRB5*01
DQB1*0303,*0601/2
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DP*0201,*0401, and HLA-DP*0401,*0402 respectively). This indicated that the majority of CD4⁺ T cell responses to HA in these donors were HLA-DR0701 restricted.

In order to test this hypothesis we repeated the HA peptide proliferation studies using a murine fibroblast line expressing HLA-DRB1*0701 (DAP.DR7.0701) as APC (Fig. 4). CD4⁺ T cell responses were seen to peptides 232–247, 417–432, 463–478, 468–483 and 508–523 indicating that these responses were DR0701 restricted. No T cell proliferative response was seen to peptide 87–102, the reason for the loss of recognition of this peptide is not clear but might reflect differences in antigen processing between murine fibroblasts and human PBMC (see Discussion).

HLA-DR0701 peptide binding studies

We were interested to examine whether the observed CD4⁺ T cell HA epitope recognition pattern was simply a consequence of dominant HA peptides having high binding affinities for HLA-DR0701, leading to their preferential recognition to the exclusion of other potential epitopes. The relative binding strength of the 118 HA peptides for purified DR0701 was estimated in a competition assay using biotinylated invariant chain peptide (Ii) (Fig. 4). Fifty seven of the peptides inhibited Ii binding with an $IC_{50} < 100$ μM. Of these nine had an $IC_{50} = 1.0$ μM and 31 had $IC_{50} = 10$ μM (Table 1). The HA peptides recognized by CD4⁺ T cells in the context of DR0701 either had detectable binding to this molecule or in the case of peptide 468–483 overlapped (by 11 residues) a peptide which was recognized in the context of DR0701 and had a relative binding strength of <10 μM for this molecule. The strongest proliferative response was associated with a peptide with $IC_{50} = 1$ μM (peptide 232–247). However, there was no direct relationship between relative binding strength and magnitude of response; the second strongest response was associated with two overlapping peptides, one with $IC_{50} = 8.1$ μM (peptide 463–478) and the other with $IC_{50} > 100$ μM (peptide 468–483), and the third strongest was associated with two peptides with $IC_{50} = 20$ μM. Only five peptides representing the HA2 subunit (residues 329–550) had $IC_{50} < 100$ μgM, with the highest relative strength belonging to peptide 508–523 (2.1 μM). In addition the peptide with the highest relative strength for DR0701 (peptide 192–207) was not recognized by CD4⁺ T cells, despite its ability to induce proliferation in the context of DR0101 [(4,5), and C. Gelder and B. Askonas, unpublished observations].

Relative binding strength of a panel of frequently recognized HA peptides for common DR alleles

In order to extend our observations on the relationship between HA peptide relative binding strength and CD4⁺ T cell recognition we investigated the binding strength of 13 HA peptides which we had previously found to be recognized in association with other HLA class II alleles (4,5). The results of this study are shown in Fig. 5. Because of the varying strength of CLIP for individual HLA-DR molecules (16), results for different HLA class II molecules cannot be directly compared. HA peptide binding was a prerequisite for CD4⁺ T cell recognition. However, peptide binding to HLA-DR did not automatically result in CD4⁺ T cell recognition. In particular, peptide 232–247, which elicits strong responses in the context of DR0701, also had measurable binding to DR0101 and to DR1302, and we have not observed CD4⁺ T cell recognition of peptide 232–247 in the context of these HLA molecules [(4,5), and C. Gelder and B. Askonas, unpublished observations].

Comparison of observed pattern of HLA-DR0701 HA peptide binding to a previously described DR0701 peptide binding motif

The observed pattern of HA peptide binding matched was compared with a reported HLA-DR0701 peptide binding motif (P1 = FILVY, P4= NST) derived from pool sequencing of peptides eluted from DR0701 (17). Though some of the HA peptides can be aligned with this motif, a new motif P1 I, L, V, (W or Y) and P9 V, I, L, (Y or F) fitted the observed pattern of HA peptide binding more exactly, and there appears to be a preference (though not an absolute requirement) for hydrophobic residues at P2 and P7, polar residues at P4 and P6, and small residues at P5 (Table 1). This putative motif suggests that though the P1 and P9 pockets are important for peptide selection, other residues in the peptide binding groove interact with DR0701 and that the final peptide affinity for DR0701 is the combination of several pocket specificities. Interestingly the few peptides which did not fit the P9 requirements have suitable residues located at P10 or P8, which might reflect some flexibility in peptide binding.

Discussion

In this report we have shown that HA-specific short-term CD4⁺ T cell lines generated from six unrelated DR0701, 1501

Fig. 1. CD4⁺ T cell recognition of HA by donors A–D. Short-term CD4⁺ T cell lines were derived before (shaded columns) and 3 months following vaccination with Influvac (dark columns). The line derived from donor C prior to vaccination was lost in culture, and the CD4⁺ T cell responses of donors E and F have previously been reported (5). Short-term lines were selected with HA A/Beijing/32/92, and T cell proliferation to HA A/Aichi/68, HA A/Beijing/32/92 and HA peptide pools tested at 14 and 21 days of culture. At 48 h T cells were pulsed with [^{3}H]thymidine and harvested 16 h later. The *x*-axis represents geometric mean of triplicate wells: control, 'Cont' = proliferative response to complete medium and autologous APC, 'Aichi' = response to A/Aichi/68 HA, 1.0 μg/ml, 'Beij' = response to HA A/Beijing/32/92, 0.1 μg/ml. Peptide pools labelled according to N-terminal residue of the first peptide in pool (five peptides per pool, 10 μg/ml each peptide). 1 = peptide pool (pp) 1–35, 25 = pp25–59, 49 = pp49–82, 72 = pp72–107, 97 = pp97–128, 118 = pp118–147, 137 = pp137–171, 161 = pp161–193, 183 = pp183–217, 208 = pp208–243, 232 = pp232–262, 252 = pp252–283, 273 = pp273–305, 295 = pp295–328, 317 = pp317–348, 333 = pp336–369, 357 = pp357–393, 384 = pp384–418, 407 = pp407–442, 432 = pp432–467, 456 = pp456–488, 478 = pp478–513, 503 = pp503–536, 526 = pp526–550. The *y*-axis represents c.p.m. Donor D differs in HLA-DQ type from the other five donors and this is associated with the non-expression of his HLA-DRB4*01 allele (see Methods).

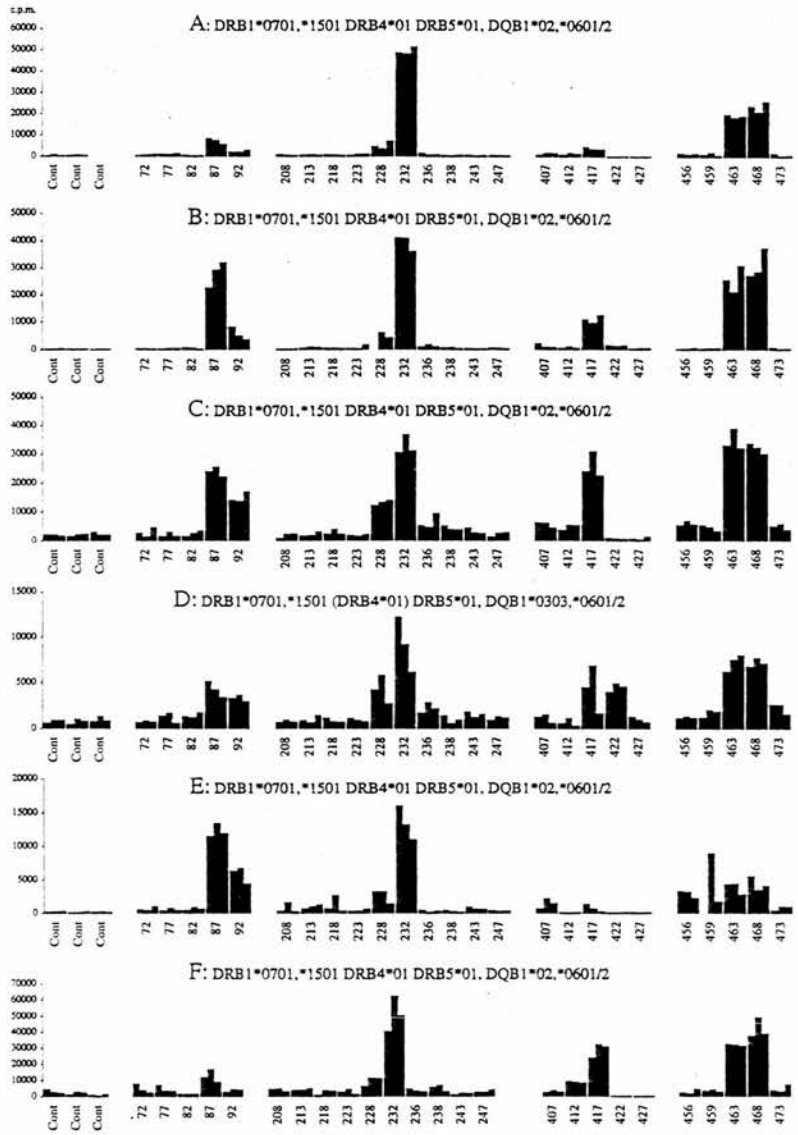

Fig. 2. CD4⁺ T cell HA responses to individual peptides within dominant HA peptide pools. T cell proliferation by donors A–F to individual 16mer peptides (5 μg/ml) within dominant peptide pools was tested at 14 and 21 days of culture using irradiated autologous PBMC as APC. 72 = peptide (p) 72–87, 77 = p77–92, 82 = p82–97, 87 = p87–102, 92 = p92–107, 208 = p208–233, 213 = p213–228, 218 = p218–233, 223 = p223–238, 228 = p228–243, 232 = p232–247, 236 = p236–251, 238 = p238–253, 243 = p243–258, 247 = p247–262, 407 = p407–422, 412 = p412–427, 417 = p417–432, 422 = p422–437, 427 = p427–442, 456 = p456–471, 459 = p459–474, 463 = p463–478, 468 = p468–483, 473 = p473–488. Data shown represents individual results from triplicate wells. Details otherwise as Fig. 1.

adults following influenza subunit vaccination principally focus on five HA peptides (peptides 87–102, 232–247, 417–432, 463–478 and 468–493) representing regions of the molecule which have been free from extensive drift mutation since the emergence of the H3N2 subtype. The observed pattern of epitope selection was HLA-DR0701, 1501 specific, as different

patterns of HA epitope recognition have been reported in the context of other HLA-DR molecules (4,5). Using DAP.DR7 cells as APC (a murine cell line which expresses HLA-DR0701) CD4⁺ T cell recognition of four of the selected peptides (peptides 232–247, 417–432, 463–478 and 468–493) was shown to be HLA-DR0701 restricted and other evidence was provided that recognition of the fifth peptide (peptide 87–102) might also be DR0701 restricted.

Using biotinylated CLIP peptide, purified DR0701 and a competition assay, the relationship between CD4⁺ T cell recognition and relative binding affinity of the HA peptides for purified HLA-DR0701 was explored and found to be complex. The relative binding affinity of the five principally recognized HA peptides varied from 0.2 (peptide 232–247) to >100 μM (peptide 468–483). Furthermore, the majority of peptides which bound to DR0701, including the peptide with the highest relative affinity for DR0701 (peptide 192–207, 0.09 μM), were not recognized by CD4⁺ T cells. The finding of peptide binding in the absence of a CD4⁺ T cell response was not unique to DR0701, as peptide 232–247 bound to both HLA-DR0101 and 1302 but was not recognized in the context of either HLA molecule.

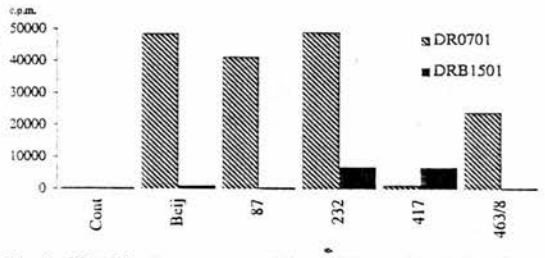

Fig. 3. CD4⁺ T cell responses to HA and HA peptides using single haplotype-matched PBMC as APC. Donor D's CD4⁺ T cell proliferation to HA A/Aichi/68, HA A/Beijing/32/92 and HA peptides was assayed at 21 days using PBMC from two partially MHC class II-matched donors as APC. 'DR0701' donor = DRB1*0101,*0701 DRB4*01, DQB1*02,*05. 'DR1501' donor = DB1*0408, *1501, DRB4*01, DRB5*01, DQB1*0301,*0601/2. Details as Fig 1.

Our finding that in adults CD4⁺ T cells principally focus on conserved HA regions contrasts the murine CD4⁺ T cell responses to HA following nasal H3 influenza A infection where both CBA (18,19) and BALB/c (18,20,21) mice principally recognize HA epitopes closely associated with the highly variable antibody neutralizing sites rather than conserved regions of the molecule (22). The murine response has also been reported to be diverse with T cell clones from individual syngeneic mice recognizing different HA epitopes and clear MHC-restricted patterns of response only emerginge once the responses of several mice are combined (23). These apparent species differences may reflect differences in previous exposure to influenza. The mice were examined after a single *in vivo* infection, whereas our adult human donors will have been repeatedly exposed to different strains influenza A virus (see below).

CD4⁺ T cell epitope selection is dependent on many factors, which include antigen processing (24,25), the ability of processed peptide to bind to MHC class II molecules (26), competition amongst peptides for MHC binding (27,28) and the presence of an appropriate repertoire of TCR (29). There are therefore several possible explanations for our finding that the relative strength of HA peptide DR0701 binding does not directly relate to the observed CD4⁺ T cell repertoire. Some HA peptides may not elicit T cell responses because their natural equivalents do not emerge from antigen processing *in vivo*. As the purity of the majority of HA peptides is not quantitated, it is possible that truncated peptides may have interfered with peptide–MHC binding. However, several of the HA peptides with high relative binding affinities for DR0701 which were not recognized, elicited strong responses in the context of other HLA class II alleles (e.g. peptides 20–35, 100–115, 105–120 and 192–207) [(4,5), and C. Gelder and B. Askonas, unpublished observations].

It is also possible that failure of some of the HA peptides to be recognized might be due to the absence of CD4⁺ T cell expressing appropriate TCR, due to regions of sequence identity between the HA peptides and self proteins. Such cross-reactivity has been demonstrated in a murine CTL clone specific for HA residues 210–219 of influenza A/Japan/57 (H2N2), which also recognized the variable region of a

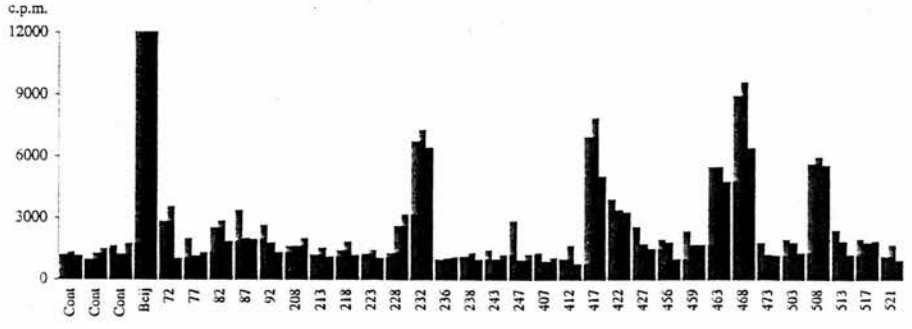

Fig. 4. HLA class II restriction studies using DAP DR7 cells as APC. HA peptide-specific CD4⁺ T cell proliferation assays were performed on a 2 week CD4⁺ T cell line selected from donor D, as described in Fig. 1, except that irradiated DAP DR7 cells (murine fibroblasts expressing HLA-DRB1*0701) were used as APC in the proliferation assays. Data shown represents individual results from triplicate wells.

Table 1. Rank order of relative HA peptide binding affinity for DR0701

Peptide	IC_{50} (μM)	Mean Proliferative response (c.p.m.)	Peptide sequence
192	0.09		T S L Y V R A S G R V T V S T K
232	0.2	30900	I Y V I V P G D I L N I N S
236	0.3		I V K P G D N T I L L A I P N R R G S G N L L
247	0.3		S T D L I L R N
188	0.3		D R D Q T S L Y V R N V S K T P G N D L I L A P R G Y F
243	0.4		L L T I A P R G Y
228	0.5	6680*	S R I S I Y W L V A E V K L W I S K K C D L E
238	0.9		K P G D I L P I N W S G R T N V S A F L L R K E R G L S S R A I T S N T L S L E
218	0.9		G S R I R W V A P V R P T G N S F T E K R R G I C S S
252	1.4		I E V K L T Q N N A T L K L I E Q L S F A I T S C F Q I
30	1.7		T N D Q I Q R D Y W I N Q S S R A I T S C D L S F R P V W A S R Q G S S F L S R Q Y Q
303	1.8		G A C P K D R Y V I L W N I S K A S S E E I K S
508	2.1	3260	K S G Y F S E R L V I N W L H S T G V C G R I C D E S
146	2.5		S F T S R L V I Q G S S R P I W S K E Y K D S
39	2.9		A T P N I Q S S R I S P S G R I C S S
213	3.2		V T I P L N I G S F A I T N S C F L L C
513	4.0		D W I L W I S K A I T N T D Q I E
20	4.2		V P N G R T S K A T S P S L E F
105	4.3		Y A S L R S L V V A T P N D Y G A R Q C P R Q T V
208	4.8		R S Q T V P N I G S R P W V
282	5.1		I T P N G V D V I P P D Y G A C S L R S Q N V
100	6.9		V I T D Y G A C P R Y V K Q N T L
300	7.1		A S G T V K L F T S T K L G C P D A S K C I T M P N G Q
198	7.7		A S G R V T K V F S D G T K L G C D A S N L S E S I M R S D A
168	7.9		M P N N G N K F D G H K Y T C G T C K S I A A D D L K S
354	8.0		R H Q N S E Y H K Y T C P C G K S C I A N A A C I
463	8.1	20100	G N G C F K S I E P Y I H K G R S N L S N E C I T M P N N
156	9.4		K S F Y I K G R N S A P P Y D V V G S
273	9.7		P I S S N C C G K S S A I N
257	9.8		Y F K I R N S T Q A A D L K S A A I M R S D A
363	9.7		Q A A D L K S T Q I N
417	12.4	13500	K I D L W S Y N A E L L V A L E
92	20.5	7000*	K A V S N C Y P P Y D D V P D Y A
87	21.2	17000*	F V E R S K A Y S N C C D P N A Y D C V
468	>100	20900	K I Y H K C D I G S I R N
Possible DR0701 peptide binding motif			L I V W Y H p h o b i c P o l a r S m a l l P o l a r H p h o b i c V L I F M W

HA peptides with a relative affinity of <10 μm for purified DR0701 are shown, plus four peptides with a lower affinity which were associated with HLA DR0701 restricted CD4⁺ T cell proliferation. Peptides with the highest affinity for DR0701 have lowest IC_{50}. Mean proliferation is geometric mean of CD4⁺ T cell responses by six donors to individual peptides, an asterisk indicates DR0701 restriction proposed on the basis of experiments with partially HLA matched APC. Sequence of HA A/Beijing/32/92 shown with sequence variation between A/Beijing/32/92 and original human H3N2 influenza A virus A/Aichi/68 underlined. Hphobic - hydrophobic.

myeloma V_H chain (30). We therefore searched the Swiss Protein Database for regions of sequence identity between the HA peptides which bound to DR0701 but were not recognized and self proteins. Interestingly we found 79% homology between the nine core residues of peptide 192–207, as predicted by our putative HLA-DR0701 peptide binding motif, and human galactosidase 2 (data not shown).

Antibody binding to an antigen has also been reported to modulate CD4⁺ T cell responses (31,32). Several HA peptides which were not recognized by CD4⁺ T cells, including peptide 192–208 which displayed the highest relative binding strength for DR0701, were located in or close to antibody neutralizing sites. The neutralizing antibody response to HA is variable between individuals and is largely influenced by exposure history (33). As the pre-vaccination exposure of these six donors varied, a more diverse pattern of CD4⁺ T cell HA recognition might be expected, if significant antibody modulation of CD4⁺ T cell recognition had occurred in this system.

Viral drift mutation may also have influenced CD4⁺ T cell HA epitope recognition. The majority of peptides with an IC_{50}

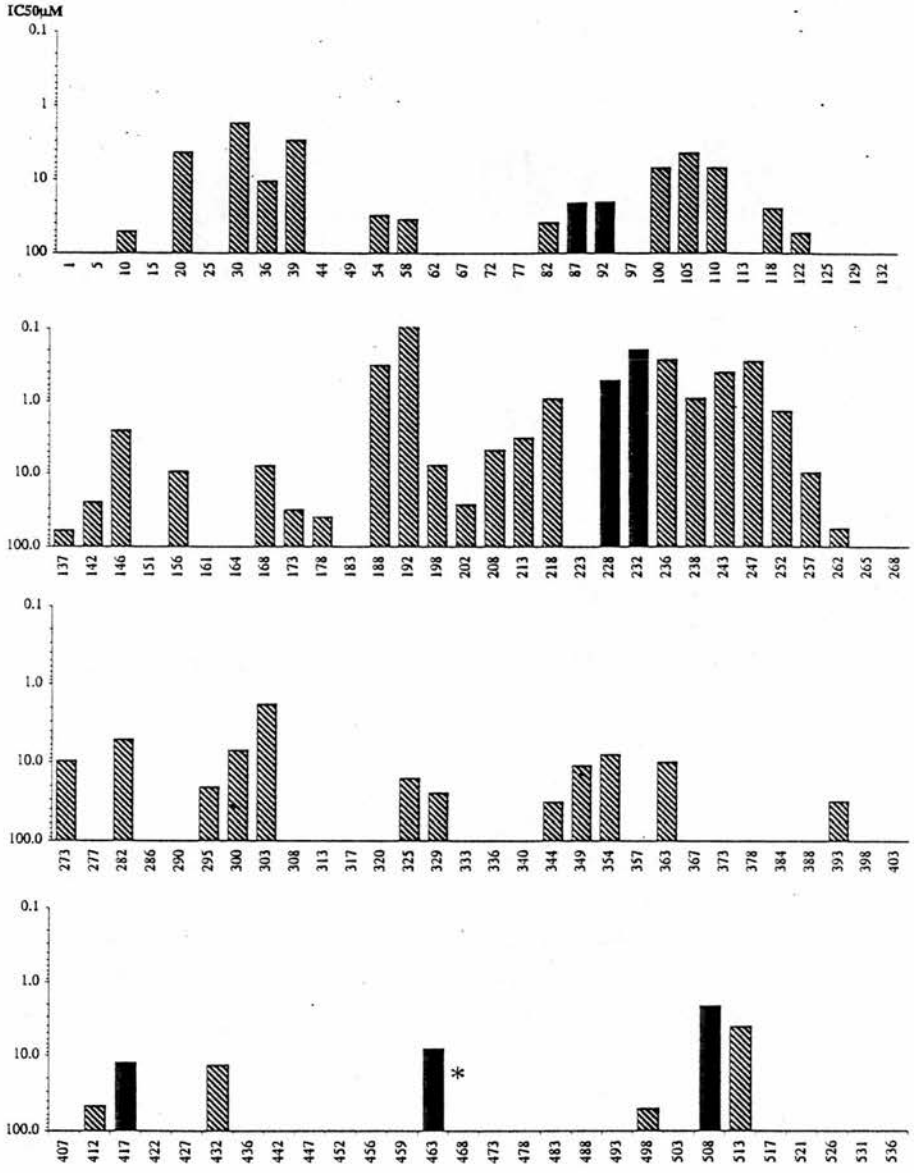

Fig. 5. Relative HA peptide binding strength for HLA-DR0701. HLA-DR0701 was incubated with biotinylated CLIP peptide with or without individual HA peptides at 37°C for 24 h at pH 5. Solutions were neutralized with Tris–HCl (pH 7.5) before transfer to wells pre-coated with L-243 antibody. Binding of biotinylated peptide was detected with avidin–horseradish peroxidase conjugate and developed with *o*-phenylenediamine. Results are presented as IC_{50} (in μM) of peptide causing 50% inhibition of invariant chain binding. The *x*-axis represents individual HA peptides, labelled by their N-terminal residue. The *y*-axis represents IC_{50} and is logarithmic, and in reverse order. Thus peptides with the highest strength (and therefore lowest IC_{50}) for DR0701 are represented by the highest columns. Filled columns indicates $CD4^+$ T cell recognition of HA peptide. An asterisk indicates $CD4^+$ T cell recognition of HA peptide with a relative binding strength for DR0701 of >100 μM.

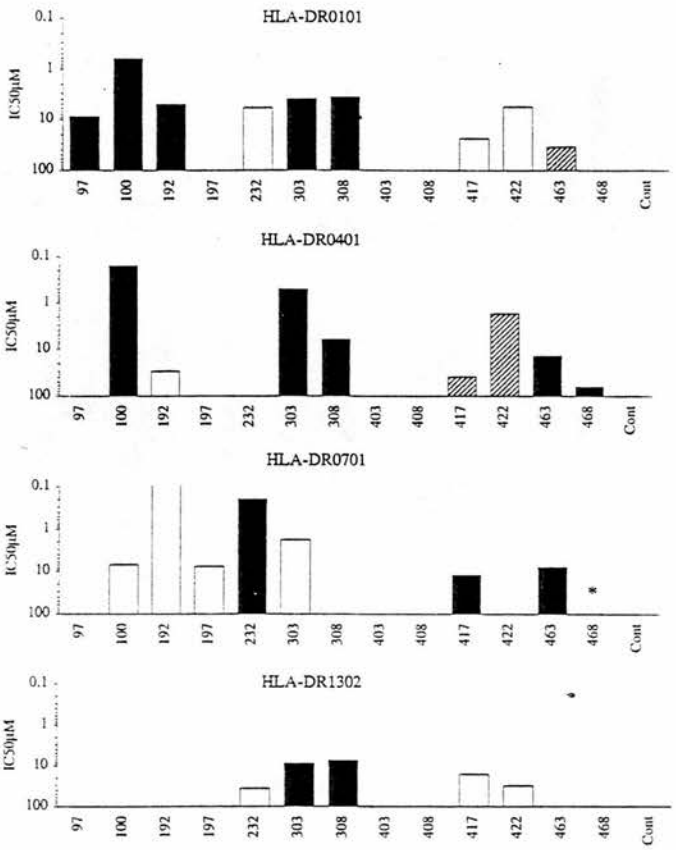

Fig. 6. Relative binding strength of 13 HA peptides for HLA-DR0101, 0401, 0701 and 1302. Binding strength of 13 frequently recognized HA peptides to three commonly expressed DR molecules was investigated using a competition assay and biotinylated CLIP peptide, results for DR0701 given for comparison. Affinities expressed as IC_{50} (in μM). A polyalanine peptide was used as a negative control, otherwise experimental details as Fig. 5. Results are not directly comparable between individual DR molecules because of differences in binding strength of CLIP for individual alleles (see text). Solid columns indicates CD4⁺ T cell proliferation in the context of a given DR molecule, clear columns indicates no response seen in the context of this DR molecule. Shaded columns indicates responses seen to the peptide pool containing this peptide, but response either not localized within the peptide pool or HLA class II restriction of CD4⁺ T cell response unknown.

for DR0701 of < 10 μM which were not recognized by the short-term CD4⁺ T cell lines represent regions of HA that have undergone drift mutations since the emergence of the H3N2 subtype in 1968 (see Table 1; N. Cox, CDC, Atlanta, GA, pers. commun.). These mutations were frequently non-conservative in nature. Six peptides representing conserved regions of HA (peptides 100–115, 105–120, 282–297, 354–369, 363–378 and 513–528) were not recognized in the context of DR0701 despite relative binding affinities for DR0701 of <10 μM. We have previously observed responses to two of these peptides (100–115 and 105–120) in the context of DR0101 [(4,5), and C. Gelder and B. Askonas, unpublished observations]. In contrast, the HA peptides which induced CD4⁺ T cell responses in the context of DR0701 contained a maximum of two highly conservative drift mutations and did not represent glycosylated regions of the molecule (see below). Thus the observed DR0701-associated HA epitope recognition pattern seen in these adult donors may have emerged as a consequence of repeated exposure to influenza A strains, differing in HA, boosting recognition of conserved regions of the molecule. Such selection of T cells specific for conserved HA epitopes would explain the apparent species differences in HA recognition described above. In order to test this hypothesis it will be important to examine CD4⁺ T cell recognition of HA by infants and children following their first exposure to influenza.

Several of the peptides which bound to DR0701 but which were not recognized, represent HA regions with potential glycosylation sites (glycosylation motif is Asn-X-Ser/Thr, peptides 20–35, 30–45, 156–171, 236–251, 238–253, 243–258, 273–288 and 282–297) (N. Cox, CDC, Atlanta, GA, pers. commun.). Glycosylation of HA has been shown in murine systems to abrogate CD4⁺ T cell recognition of HA (34) and therefore may account for the absence of CD4⁺ T cell recognition of our synthetic peptides despite their high relative binding affinities for DR0701. We have only rarely seen significant T cell recognition (5 times the geometric mean of background response to medium and APC) of peptides representing glycosylated regions of HA in the context of any HLA-DR molecule examined to date (DR0101, 0102, 0103, 0301, 0401, 0402, 0408, 0701, 0801, 1001, 1101, 1201, 1301, 1302, 0101, 0102, 0103, 0301, 0401, 0402, 0408, 0701, 0801, 1001, 1101, 1201, 1301, 1302, 1501) [(4,5), and C. Gelder and B. Askonas, unpublished observations]. It will be important to examine whether human CD4⁺ T cells are able to recognize glycosylated synthetic peptides representing these regions.

Finally these results illustrate the difficulty in accurately predicting CD4⁺ T cell epitopes in viral antigens on the basis of peptide binding studies alone.

In conclusion we have demonstrated the precise control HLA class II exerts over CD4⁺ T cell HA epitope selection in unrelated adults. Immunodominance appears not to be directly related to the relative strength of HA peptide–HLA class II binding, and thus reflects complex interactions between antigen processing, intracellular competition for HLA binding, TCR repertoires and repeated exposure to different strains of influenza A viruses.

Acknowledgements

We thank the following for their contributions to this study: Dr R. Brands and Solvay Duphar for the gift of HA A/Beijing/32/92, Dr G. Plant for the gift of LBF, H. Reyburn for assistance growing LBF, Dr N. Cox (CDC, Atlanta, GA) for unpublished sequence information on HA A/Beijing/32/92, Professor R. Lechler (RPMS, London) for the gift of DAP DR7.0701 cells, Dr J. Skehel (NIMR, London) for the gift of HA A/Aichi/68, Professor D. Wraith (University of Bristol) for advice on peptide synthesis, and Professor C. Bangham (Imperial College London), Professor L. K. Borysiewicz and Dr S. Man (UWCM, Cardiff) for helpful discussions. This work was supported by the Wellcome Trust. C. M. G. is a Wellcome Trust Advanced Training Fellow. M. P. D. is a Wellcome Trust Prize Student and was supported by the Lionel Murphy Foundation. A. V. S. H. is a Wellcome Trust Principal Fellow.

Abbreviations

APC	antigen-presenting cell
HA	haemagglutinin
HLA	human leukocyte antigen
PBMC	peripheral blood mononuclear cells

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Short paper

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Prediction and identification of an HLA-DR-restricted T cell determinant in the 19-kDa protein of *Mycobacterium tuberculosis*

An allele-specific motif has been identified in the sequence of several peptides which are recognized by T cells in association with HLA-DR1. In order to test the predictive values of such a motif we analyzed the 19-kDa antigene from *Mycobacterium tuberculosis* and identified a sequence containing a pattern characteristic of DR1 restriction. Peripheral blood mononuclear leukocytes from every DR1 and 4 individual tested responded to the corresponding synthetic peptide. Nine other donors, constituting seven different DR alleles, failed to recognize this sequence. Recognition of the peptide in association with DR1 and DR4 was confirmed using T cell clones and transfected murine L cell lines expressing DR molecules.

1 Introduction

The induction of the cellular immune response is inititated when an appropriate T cell receptor recognizes the complex formed between fragments of protein antigens and molecules encoded by the major histocompatibility genes [1]. Recent experiments have demonstrated that the genetic basis of antigen-specific immune responsiveness can be explained by the ability of a particular major histocompatibility complex (MHC) allele to bind a fragment of the immunizing protein or pathogen [2]. Understanding the structural basis of this genetic restriction is important in selecting antigens for use both as diagnostic reagents and/or components of potential vaccines. The identification of peptide antigens with similar structural features restricted by the same MHC class II molecule has suggested the presence of allele-specific subpatterns in T cell epitopes [3]. In the case of the HLA class II allele DR1, a cluster of a positively charged amino acid and three hydrophobic residues in relative positions 1, 4, 5 and 8 is common in two defined determinants and appears to be necessary for this peptide to interact with the restriction element [4]. If the peptides adopt a helical conformation then these discontinuous residues would be juxtaposed to form a common facade of the helix allowing the peptides to selectively bind DR1.

To test the predictive value of this template, we scanned the sequence of the 19-kDa protein of mycobacteria for the presence of the motif. The protein was chosen because T cells specific for it are well represented in both the human [5, 6] and murine [7] repertoires reactive with mycobacteria. The peptide corresponding to the only area containing the motif in the protein sequence was shown to be recognized by DR1 and DR4-restricted T cells.

[I 6780]

△ Supported in part by the Foundation for Medical Research grant 900-509-109, the Immunology of Leprosy component of the United Nations Development Program/World Bank/World Health Organization Special Programme for Research and Training in Tropical Diseases and the Netherlands Leprosy Relief Association.

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Abbreviations: **EBV:** Epstein-Barr virus **IL 2:** Interleukin 2 **MHC:** Major histocompatibility complex **MTSE:** *Mycobacterium tuberculosis* soluble extract **PBMC:** Peripheral blood mononuclear cells

2 Materials and methods

2.1 Antigen preparations

Mycobacterium tuberculosis soluble extract (MTSE) was prepared by mechanical distruption of *M. tuberculosis* H37Rv grown as a surface pellicle on Sauton's medium. The recombinant 19-kDa (clone Y4147) and λgt11 antigen preparations were prepared as described previously [5]. Purification of the 19-kDa protein by affinity chromatography using antibody TB23, and sequencing of the DNA insert from a λgt11 clone (Y3239) which expresses the 19-kDa antigen are described elsewhere (Lathigra et al., manuscript in preparation). The 19-kDa peptide (see Table 1, Sect. 3) was synthesized by using solid-phase techniques [4].

2.2 Isolation of human T cell clones

Peripheral blood mononuclear leukocytes (PBMC; 2.5×10^5/ml) were stimulated in RPMI 1640 medium supplemented with 10% screened human A^+ serum, 2 mM L-glutamine and 100 IU/ml penicillin/streptomycin with 1.0 μg/ml of affinity-purified 19-kDa protein. Activated lymphocytes were isolated on Ficoll-Paque (Pharmacia, Uppsala, Sweden) and cloned by limiting dilution (0.3 blasts/well) in the presence of autologous irradiated PBMC mixed (4 : 1) with Epstein-Barr virus (EBV)-transformed B cells (total cell number 5×10^5/ml), interleukin 2 (IL 2; Lymphocult-T, Biotest Serum Institut GmbH, Frankfurt, FRG) and affinity-purified 19-kDa protein (1.0 μg/ml) in Terasaki plates (Nunc, Roskilde, Denmark) as described previously [5, 8]. Growing clones were transferred at day 7 to 96-well flat-bottom microtiter plates and subsequently to 24-well plates. At each transfer the clones received filler cells, antigen (MTSE;, 1 μg/ml) and IL 2. The clones were expanded by the addition of fresh IL 2 every 3–4 days and filler cells together with antigen every 7 days. Prior to use

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in proliferation assays the T cell clones were rested 6–8 days after the last addition of filler cells.

2.3 Proliferation assays

Cloned T cells (5×10^4/ml) were stimulated with antigen in the presence of irradiated autologous EBV-transformed B cells (10^5/ml) or mitomycin C-treated transfected murine L cells [4]. After 60 h incubation tritiated methyl thymidine ([^{3}H]dThd; 1 μCi/well: Amersham International, Amersham, Bucks., GB) was added and the cultures were harvested onto glass-fiber filters 8–16 h later. The duration of unfractionated PBMC cultures (1×10^5) was 7 days. Proliferation as correlated with [^{3}H]dThd incorporation was measured by liquid scintillation spectroscopy. The results are expressed as mean counts per minute (cpm) ± % standard error of the mean for triplicate cultures.

2.4 Transfected murine L cells

Full length cDNA clones encoding HLA-DR1β and DR4(Dw15)β were subcloned together with a DRα cDNA clone into an SV40-based cDNA vector (Ikeda, H., manuscript in preparation). These constructs were transfected into mouse Ltk$^-$ cells using the calcium phosphate technique [9] and transfectants selected with hygromycin B (Calbiochem/Behring Diagnotics, La Jolla, CA) at a concentration of 1 mg/ml. Transfectant cell lines expressing high levels of the surface expression of DR1 and DR4 products were isolated by flow microfluorimetric sorting. The HLA-DR7 expressing transfectants have been described previously [10].

2.5 HLA typing

HLA-DR and DQ typing was performed on PBMC using 80 sera and the two-color fluorescence test [11].

3 Results and discussion

A peptide corresponding to the first 15 amino acids of the predicted sequence of the 19-kDa protein of *M. tuberculosis* was synthesized and tested for its ability to be recognized by human helper T cells (Table 1). This particular region was chosen because it contained a similar pattern of amino acids previously identified in DR1-restricted T cell epitopes from influenza virus [4]. PBMC, isolated from either *Bacillus Calmette Guérin* (BCG)-vaccinated individuals or tuberculosis patients, were screened for their ability to proliferate in response to whole bacterial extract or the peptide. As predicted, lymphocytes from each DR1 individual tested responded to the peptide (Table 2). Individuals expressing DR4 gene products also recognized this sequence (Table 2). The ability of DR1 and DR4 class II molecules to present the identical peptide has been observed previously for influenza virus-reactive T cell clones [12], and can be accounted for by sequence and structural similarity in their antigen-combining sites ([13], Rothbard et al., manuscript in preparation). Only two out of the 11 donors lacking DR1 and/or DR4 (LB14) responded to this peptide, of which one was DR13, 14 while the other was a DR3 homozygote. However, other individuals expressing each of these alleles failed to respond to the peptide.

Table 1. Structural similarity of the amino terminal peptide of the 19-kDa protein of mycobacteria to known HLA-DR1-restricted peptides[a)]

Influenza hemagglutinin 307-319	P-K-Y-V-K-Q-N-T-L-K-L-A-T
Influenza matrix 17-29	S-G-P-L-K-A-E-I-A-Q-R-L-E
M. tuberculosis 19-kDa 1-15	E-H-R-V-K-R-G-L-T-V-A-V-A-G-A

a) Amino acids represented by the single letter code. The underlined residues identify the presence of the motif associated with HLA-DR1 restricted T cell epitopes [3, 4]. The 19-kDa peptide sequence is derived from the open reading frame identified in the DNA insert from a λgt11 recombinant clone Y3293 that codes for 19-kDa protein of mycobacteria (Lathigra et al., manuscript in preparation).

The results of the panel study were confirmed by examining the specificity and restriction of T cell clones isolated from one of the donors (PTER) by growing the lymphocytes in the presence of affinity-purified 19-kDa protein. Of the two clones specific for the recombinant antigen isolated, one (AP1.17) was specific for the 15 amino acid peptide (Table 3), demonstrating that this peptide is naturally immunogenic.

The restriction specificity of clone AP1.17 was analyzed using murine L cells expressing products of transfected HLA-DR genes to present the peptide antigen (Table 4). Transfectants were selected as the source of antigen-presenting cells because they express only the products of the transfected genes in isolation from other MHC class II species. Murine L cells expressing DR1, compatible with the HLA-DR serotype of the donor (Table 2), were able to induce antigen-dependent proliferation of clone AP1.17 unlike the control DR7$^+$ L cell. This confirmed the results of the population study that recognition of the 19-kDa peptide was DR restricted. Furthermore, L cells expressing DR4(Dw15) also were able to repesent the peptide.

The analysis of peptides capable of stimulating DR1-restricted influenza virus-immune T cell clones has identified a sequence motif involved in the binding of peptide in combining site of DR1 molecules [4]. The experiments described in this study demonstrate that this motif can be used predictively to identify regions of proteins capable of interacting with DR1 and DR4dw15. The results also imply that the three peptides, the two from influenza and the peptide from the 19-kDa protein, interact with similar residues in both DR1 and DR4. In addition, the T cell receptors on each of the antigen-specific clones must be capable of binding each peptide in the context of the two MHC alleles. Modelling the class II sequences based on the HLA-A2 structure reveals that the proposed antigen-combining site is composed of residues of both the conserved α chain and the polymorphic β chain [14, 15]. In addition, the upper facade of the helix of the β chain is identical between DR1 and DR4dw15 [4]. There are nonconservative changes in the residues that form the bottom of the binding site in the two alleles, but whether they are sufficiently similar to allow identical peptides to bind both sites or whether the peptide does not directly interact with them remains to be determined. We are currently analyzing the ability of mutants of these three peptides to be recognized by T cell clones in the context of

Table 2. Polyclonal T cell responses to the peptide to the 19-kDa peptide[a)]

Donor	HLA class II phenotype DR				DQ		Response (Δcpm) MTSE	19-kDa peptide
	LB14	w52	w53	w1	w2	w3		
46wZ							56976	9041
1023							20857	8327
PHER							6Z414	9735
864wZ							56747	5631
374w6							229138	10462
HSP							164988	13309
236w2							59521	9242
IWIN							61997	10676
DEAS							26359	7984
700w8							149205	14708
236w6							40398	0
655w8							50470	139
883w8							21898	1207
BAGHE							62822	0
976w4							11547	2297
976w1							57909	455
742w2							13638	0
426wZ							45541	1207
267wx							24776	101

a) The results are expressed as Δcpm where the medium backgrounds (mean < 250 cpm) are subtracted from the response to antigen. Standard errors were less than 25%. LB14 is a newly defined DR specificity which is associated with DR1, 4 and DRw10 and is closely related with, if not identical to, MC1 [14].

Table 3. Antigen specificity of T cell clone induced with the 19-kDa protein[a)]

Antigen	Proliferative response (cpm ± % SEM) AP1.17
MTSE	7149 (2)
Affinity-purified 19-kDa protein	5574 (5)
I-15 of 19-kDa protein	4994 (17)
Recombinant 19-kDa protein, Y4147	3850 (11)
λgt11	886 (7)
Medium	714 (7)
IL 2	2695 (9)

a) T cell clones (5×10^4/ml) were stimulated with autologous irradiated EBV-transformed B cells (5×10^4/ml) in the presence of MTSE (1 μg/ml), affinity-purified 19-kDa (1 μg/ml), 19-kDa peptide (10 μg/ml), 19-kDa recombinant antigen (Y4147; 10 μg/ml) and λgt11 (10 μg/ml). Proliferation was determined by [^{3}H]dThd incorporation at 72 h. The results are expressed as mean cpm ± % SEM for triplicate cultures.

Table 4. Proliferative response of cloned T cells to the 19-kDa peptide presented by murine L cells expressing HLA-DR1 or DR4[a)]

T cells	Accessory cells	Antigen	Response (cpm ± % SEM)
AP1.17	—	—	127 (17)
+	—	19-kDa	203 (19)
+	EBV-B	—	431 (9)
+	+	19-kDa	8719 (3)
+	DRα:DR1β L cells	—	162 (31)
+	+	19-kDa	6338 (20)
+	DRα:DR4β L cells	—	357 (12)
+	+	19-kDa	3822 (8)
+	DRα:DR7β L cells	—	112 (27)
+	+	19-kDa	305 (3)

a) T cells of clone AP1.17 (5×10^4/ml) were stimulated with and without 19-kDa peptide (1 μg/ml) in the presence of irradiated autologous EBV-transformed B cells (10^5/ml) or mitomycin C-treated transfected murine L cells. Proliferation was determined as described in the legend to Table 3.

DR1 and different DR4 alleles to determine the precise orientation of the peptides in the binding site.

We should stress that not all peptides with this 4 amino acid motif will bind DR1 because these are not the sole contacts between the MHC protein and the peptide [4]. Further experiments are required to refine and expand the motif to include all the residues needed for DR1 binding. Also, we do not believe that all DR1-restricted peptides will be recognized by DR4. Even though these three examples appear to bind in a similar fashion, other peptides could have different orientations in the binding site. Nevertheless, the ability of a given short peptide to bind to more than a single MHC class II protein has practical applications in the development of vaccine components, as will the identification of similar predictive motifs for other HLA-DR alleles.

The authors thank Drs. R. Lathigra and R. Young for communicating the sequence of the 19-kDa protein, Dr. I. Schreuder and colleagues for HLA typing and Dr. M. Contreras, National Blood Transfusion

Centre, Edgware, for providing the blood donors. We also thank Drs. Lechler, D. Young, J. Trowsdale and J. Ivanyi for helpful discussions.

Received April 19, 1988.

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Announcements

International Meeting on Opportunistic Infections

Paris
November 24–26, 1988

Review lectures, round tables and poster presentations on interactions between specific pathogens and the immunocompromized host and on some clinical and therapeutical aspects of opportunistic infections.

Registration and abstract submission to Dr. N. Wierzbicki, Fondation Franco-Allemande, LTM/Sarbach – BP 22, F-92151 Suresnes Cedex, France.

Third Banff Conference on Reproductive Immunology

The Molecular and Cellular Immunobiology of the Maternal Fetal Interface
Banff Conference Center
Banff, Alberta, Canada
January 12–14, 1989

The conference organizers will be Thomas G. Wegmann and Thomas J. Gill III. Those interested in attending, and presenting a poster if desired, should contact Dr. Thomas J. Gill III, Department of Pathology,University of Pittsburgh School of Medicine, Pittsburgh, PA 15261. The registration fee will be US $ 150.

Micronutrient Effects on Immune Functions

Omni Park Central, New York
June 1–2, 1989

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Conference chair persons:

Ranjit, K. Chandra, M.D., F.R.C.P.(C), Department of Pediatrics, Memorial University of Newfoundland, St. Johns, Newfoundland, Canada.

Adrianne Bendich, Ph.D., Clinical Nutrition, Hoffmann-LaRoche Inc., Nutley, NJ, USA.

There will be a contributed poster session. The deadline for submission of abstracts (200 words or less) is **February 1, 1989.** Send abstracts to: Dr. Adrianne Bendich, Clinical Nutrition, Hoffmann-LaRoche Inc., 340 Kingsland Avenue, Nutley, NJ 07110-1199, USA (Abstract form is not necessary).

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The European Journal of Immunology does not publish book reviews; books submitted for review will not be returned.

Overlapping T-cell epitopes in the group I allergen of *Dermatophagoides* species restricted by HLA-DP and HLA-DR class II molecules

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The induction of IgE antibodies reactive with the group I allergen of Dermatophagoides *species (house dust mite [HDM]), which comprise a major component of the allergic immune response in HDM-atopic individuals, is dependent on the functional activity of specific CD4+ T cells. In this report we demonstrate that for a particular HDM-atopic individual the T-cell response to the group I allergen of* Dermatophagoides pteronyssinus *(*Der p *I) is limited to a single region (residues 101-143) of the protein. By mapping the fine antigen specificity with T-cell clones, we observed that the sequence 101-131 of* Der p *I contains a cluster of at least three overlapping T-cell epitopes. Analysis of the HLA class II restriction specificity of the T-cell clones revealed that the T-cell epitope, residues 110-131, was restricted by HLA-DRB1*0101. In contrast, peptide* Der p *I, 110-119 was recognized in association with HLA-DPB1*0402. However, the ability of cloned T cells to proliferate to the peptide* Der p *I, 107-119 presented by HLA-DPB1*0401, HLA-DPB1*0402, and HLA-DPB1*0501 expressing accessory cells illustrates the heterogeneity of the restriction specificity of this region of* Der p *I. The application of this information in the design of peptide-based immunotherapy in the management of allergic responses to HDM is discussed. (J ALLERGY CLIN IMMUNOL 1994;93:891-9.)*

Key words: Peptides, house dust mite, Der p *I, HLA-DR, HLA-DP, T-cell epitopes*

In genetically susceptible individuals, exposure to proteins derived from mites of the genus *Dermatophagoides* (*D. pteronyssinus* and *D. farinae*) results in clinical symptoms ranging from extrinsic asthma and allergic rhinitis to atopic dermatitis. Serologic analysis with IgE antibodies from patients with house dust mite (HDM) allergy suggests that multiple antigens are present in HDM extracts.[1] Nevertheless, it appears that a major component of the humoral immune response comprises antibodies reactive with the group I allergens of HDM (*Der p* I and *Der f* I).[2, 3] The results of several studies suggest that in excess of 50% of anti-HDM IgE antibodies are specific for *Der p* I and are found in approximately 70% of sera from allergic individuals.[4, 5]

Abbreviations used

APCs:	Antigen-presenting cells
EBV:	Epstein-Barr virus
HDM:	House dust mite
PBMCs:	Peripheral blood mononuclear cells

The induction of specific IgE antibodies and the activation of polymorphonuclear granulocytes, both of which contribute to inflammatory responses to HDM allergens, are dependent on CD4+ T cells.[6-8] Therefore in the development of specific immunotherapy, targeted at the CD4+ T-cell population, it is essential to define in detail the antigen and HLA class II restriction specificity of the HDM-reactive T-cell repertoire in HDM-atopic individuals. In the peripheral blood

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Supported by grants from the Wellcome Trust; the Medical Research Council, England; and Tripos Associates.

Robyn O'Hehir is the recipient of a Wellcome Senior Research Fellowship in Clinical Sciences.

Received for publication Mar. 25, 1993; accepted Sept. 23, 1993.

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0091-6749/94 $3.00 + 0 1/1/52640

TABLE I. HLA-D region specificities of the panel of EBV-transformed B cells used as APCs

Cell line	HLA-DR specificities		HLA alleles							
			DRB1	DRB3	DRB4	DRB5	DQA1	DQB1	DPA1	DPB1
HOM2	DR1 Dw1		0101	–	–	–	0101	0501	ND	0401
PGF	DR15 Dw2		1501	–	–	0101	0102	0602	01	0401
PMG075	DR1 Dw20		0102	–	–	–	0101	0501	01.02	0301,0401
TISI	DR11	DR52 Dw25	1103	0202	–	–	0501	0301	01	0402
WT24	DR16(2) Dw21		1601	–	–	0201	0102	0502	01	0301
YAR	DR4 Dw10		0402	–	0101	–	0301	0302	01	0401

of HDM-allergic individuals T cells that proliferate in response to stimulation with extracts of HDM have been identified.[9-12] Furthermore, with electrophoretically separated and affinity-purified allergen preparations, T-cell responses to *Der p* I have been demonstrated.[13] However, now that the complementary DNA encoding *Der p* I has been cloned, allowing the primary amino acid sequence to be derived from the nucleotide sequence,[14] it is possible to analyze the specificity of the T-cell repertoire reactive with *Der p* I with the use of truncated recombinant proteins and synthetic peptides. When this approach to analyze the antigen specificity of *Der p* I–induced T-cell clones was used, four regions of the protein (residues 45-67, 94-104, 101-119, and 117-143) that contain T-cell epitopes were identified.[15, 16] Furthermore, the HLA class II restriction specificity of these T-cell clones was determined and revealed that both HLA-DR– and HLA-DP–encoded gene products are functional in the presentation of *Der p* I.

It is important to identify the immunodominant sites in *Der p* I that are recognized by T cells from atopic individuals. The information derived from these studies will be of value both in enhancing our understanding of the role of T cells in allergic inflammation and in the development of allergen-based immunotherapy. This prompted us to investigate in detail, with the use of a T cell line and clonal analysis, the diversity of the antigen and HLA class II restriction specificity of the T-cell repertoire reactive with *Der p* I, a major allergen of HDM. We observed that for a particular atopic HDM-allergic individual the anti-*Der p* I T-cell response was directed exclusively toward the region of the protein spanning amino acids 101-143. When the fine specificity of T-cell clones from this donor was mapped, it appeared that within a region of *Der p* I encompassing amino acids 101-131 there is a cluster of overlapping T-cell epitopes. Furthermore, from the patterns of proliferation when allergen was presented by different HLA class II molecules, it appeared that the T-cell epitope spanning residues 110-131 was restricted by HLA-DRB1*0101, whereas residues 107-119 and 110-119 were recognized in association with HLA-DPB1*0402. T cells specific for the peptide *Der p* I, 107-119 and able to respond to peptide in association with HLA-DPB1*0401, HLA-DPB1*0402 and HLA-DPB1*0501 were cloned from the peripheral repertoire of the HDM-allergic individual.

METHODS

Antigens

Lyophilized extracts of *D. pteronyssinus* and *D. farinae* were supplied by SmithKline Beecham (Epsom, Surrey, England). Peptides derived from nucleotide sequences of *Der p* I and *Der p* II were synthesized in our department as previously described.[15] The peptides were the generous gift of Dr. M-C. Kuo, ImmuLogic Pharmaceutical Corporation, (Waltham, Mass.) or were purchased from Chiron Mimotopes Ltd. (Clayton, Victoria, Australia).

Antibodies

The monoclonal antibodies L243 (anti-HLA-DR)[17] and B7/21 (anti-HLA-DP)[18] were isolated from the culture supernatants of the respective hybridoma cell lines and purified with protein A Sepharose (Pharmacia, Uppsala, Sweden).

Isolation of human HDM-reactive clones

Der p I–reactive T-cell clones were isolated from an HDM-allergic individual by limiting dilution cloning as described previously.[10] Peripheral blood mononuclear cells (PBMCs) (10^6/ml) from an HDM-atopic individual with perennial rhinitis were stimulated with an optimal concentration of *D. pteronyssinus* (20 μg/ml) for 7 days in RPMI-1640 medium supplemented with 2 mol/L L-glutamine, 100 IU/ml penicillin/streptomycin (Gibco Life Technologies, Paisley, Scotland) and 5% screened, inactivated human A+ serum (National Blood Transfu-

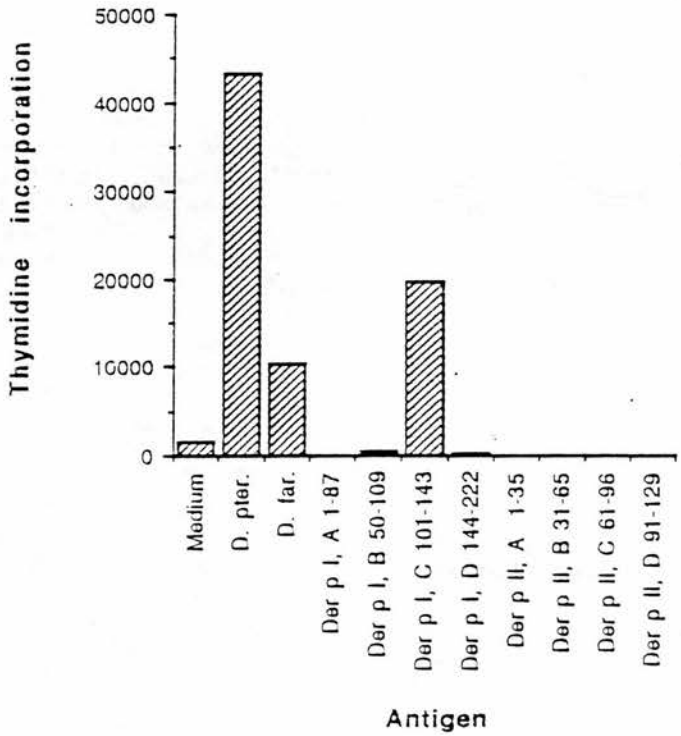

FIG. 1. Antigen specificity of *D. pteronyssinus*–induced T cell line. T cells from an HDM-atopic individual were stimulated with extracts of *D. pteronyssinus* and *D. farinae*, peptide pools were derived from *Der p* I and *Der p* II (5 μg/ml) in the presence of APCs, and proliferation was measured.

sion Service, Edgeware, England). A long-term line of one of the donors was established by enriching lymphoblasts on Ficoll-Paque (Pharmacia) in the presence of irradiated autologous PBMCs (5×10^5/ml; 2500 rad), *D. pteronyssinus* (20 μg/ml) and interleukin-2 (10% vol/vol; Lymphocult T, Biotest Folex, Frankfurt, Germany). Viable cells from the various lines (0.3 cells/well) were cloned by limiting dilution in supplemented medium and plated in Microtest II plates (Nunc, Gibco Life Technologies) together with irradiated autologous PBMCs, *D. pteronyssinus*, and interleukin-2. After 7 days, growing clones were transferred to flat-bottom 96-well microtiter plates and subsequently to 24-well plates. The clones were maintained with interleukin-2 every 3 to 4 days and irradiated autologous PBMCs and *D. pteronyssinus* every 7 days. In all experiments clones were tested 7 to 8 days after the last addition of antigen and antigen-presenting cells (APCs).

Proliferation assays

Cloned T cells (10^5 cells/ml) were stimulated with peptides (0.1 to 10.0 μg/ml) in the presence of APCs. The APCs used in the experiments were irradiated (2500 rad) autologous PBMCs (10^5 cells/ml), irradiated (5000 rad) Epstein-Barr virus (EBV)–transformed B cells (10^5 cells/ml) (Table I), or mitomycin C–treated murine fibroblasts expressing HLA-D region gene products (10^5 cells/ml). The murine fibroblasts (DAP3), transfected with DRB1*0101, DPB1*0401, DPB1*0501, and DPB1*0402 genes were kindly provided by Dr. John Trowsdale, ICRF, London; Dr. Sandra Rosen-Bronson, Georgetown University Medical School, Washington D.C.; and Dr. Hidetashi Inoko, Tokai University School of Medicine, Bosheida, Japan, respectively. In serologic inhibition assays antibodies were added over a concentration range at the initiation of cultures. After 60 hours of incubation the cultures were pulsed with tritiated methyl thymidine (1 μCi/well; Amersham International Inc., Amersham, England), and the cultures were harvested 8 to 16 hours later. Proliferation as correlated with tritiated methyl thymidine incorporation was measured by liquid scintillation spectroscopy. The results are expressed as mean counts per minute for triplicate cultures. The standard error of the mean for all experiments was less than 20%.

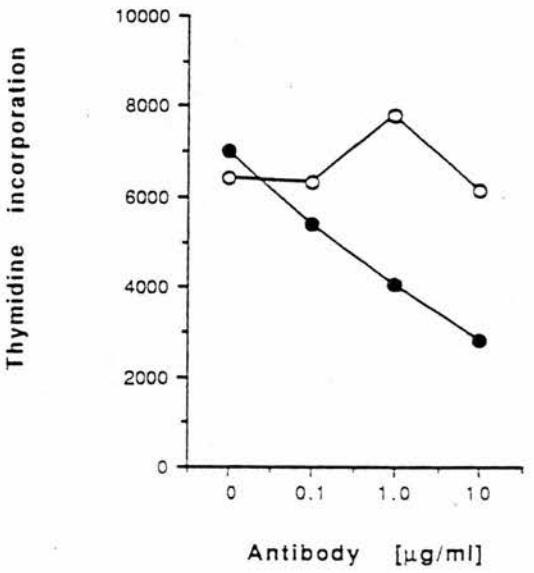

FIG. 2. Anti-HLA-DP class II antibody inhibits antigen-dependent proliferative response of *Der p* I–reactive T cell line. The inhibitory effects of anti-HLA-DP (B7/21) (•) and HLA-DR (L243) antibodies (○) were determined in proliferation assays with the T cell line, APCs, and *D. pteronyssinus* (20 μg/ml).

RESULTS

Antigen and major histocompatibility complex class II restriction specificity of *D. pteronyssinus*–induced T cell line

The antigen specificity of a long-term T cell line expanded from PBMCs isolated from an HDM-atopic individual was analyzed with pooled peptides derived from the sequences of *Der p* I and *Der p* II. Although proliferation to each of the peptide pools was determined over a range of concentrations (0.1 to 50 μg/ml) the T cell line responded to only *Der p* I 101-143 (peptides 101-119, 110-131, 111-134, and 120-143) (Fig. 1). Furthermore, the T cell line responded to both *D. pteronyssinus* and *D. farinae* extracts, suggesting the presence of T cells reactive with conserved sequences located between residues 101-143 of *Der p* I and *Der f* I. The ability of the murine monoclonal anti-HLA-DP (B7/21) antibody but not the anti-HLA-DR (L243) antibody to inhibit antigen-induced proliferation in a dose-dependent manner (Fig. 2) suggests that the major component of the T cell line responds to allergen presented in association with HLA-DP class II molecules.

Antigen specificity of *Der p* I–reactive T-cell clones

The T cell line was cloned by limiting dilution, and three T-cell clones were screened for their antigen specificity with overlapping peptides of *Der p* I (Fig. 3, *A-C*). T cells of clone KS7.4 were screened with a nested set of overlapping peptides covering residues 103-125. Peptides 105-119 and 110-124 stimulated the T cells, whereas peptides 105-118 and 111-125 both failed to induce proliferation (Fig. 3, *A*), suggesting that the minimal T-cell epitope recognized by KS7.4 is residues 110-119. In a previous report we demonstrated that T cells of clone KS2.12 respond to residues 101-119, defining the importance of Ile at position 119.[15] Now with peptides truncated from the amino terminus (residue 106) it is apparent that residues 107-119 contained the optimal T-cell epitope recognized by the T-cell clone KS2.12 (Fig. 3, *B*). The T-cell clone KS2.15 responded to peptides 101-143 and 110-131 but not to peptides 107-118, 123-136, or 126-139 (Fig. 3, *C*) when tested over a concentration range. Thus KS2.15 recognizes a region of *Der p* I between residues 110 and 131. Unlike the T cell line from which they were isolated, none of the T-cell clones were cross-reactive with *D. farinae* (data not shown).

HLA-D region restriction specificity of *Der p* I T-cell clones

In order to determine the HLA-D region restriction specificity, the T-cell clones were stimulated with antigen in the presence of either HLA-typed EBV-transformed B cell lines (Table I) or murine fibroblasts expressing HLA-D region molecules. A dose-dependent proliferative response of T-cell clone KS7.4 was induced to antigen presented by fibroblasts expressing HLA-DPB1*0402 but not HLA-DPB1*0401 (Fig. 4, *A*). Similarly, the untransfected fibroblast line (DAP 3) and fibroblast lines expressing HLA-DRB1*0101 failed to present antigen. The EBV-transformed B-cell line (WT24) induced proliferation in the presence of high concentrations of antigen but was markedly lower than that mediated by autologous EBV-transformed B cells (KSE; Fig. 4, *A*). Previous investigation of the HLA class II restriction pattern of KS2.12 demonstrated that T cells derived from this clone recognized antigen in association with HLA-DPB1*0401.[15] Extension of this analysis with murine fibroblasts expressing different alleles of HLA-DP revealed that HLA-DPB1*0401, DPB1*0402, and DPB1*0501-

expressing fibroblasts all presented peptide to KS2.12, although DPB1*0501-positive cells were less efficient (Fig. 4, *B*). No response to antigen was observed in the presence of DRB1*0101-positive fibroblast transfectants. Comparison of the primary amino acid sequences indicates that HLA-DPB1*0401, HLA-DPB1*0402, and HLA-DPB1*0501 differ at positions 31, 38, 57, 58, 86, 87, 88, and 89 (Fig. 5).

The T-cell clone KS2.15 proliferated to HDM allergen presented by the EBV-transformed B cells, KSE and HOM2 (Fig. 4, *C*). In contrast, the B cell lines TISI, PGF, PMG075, and YAR all failed to induce antigen-dependent proliferation. The B cell line WT24 was able to stimulate KS2.15 but only in the presence of high concentrations of antigen and was suboptimal compared with KSE or HOM2 (Fig. 4, *C*). Comparison of the pattern of proliferation with the HLA class II haplotypes of the EBV-transformed B cells (Table I) suggests that KS2.15 is restricted to HLA-DRB1*0101.

Molecular models of T-cell epitopes mapped on a computer model structure for the *Der p* I molecule

The T-cell epitopes for HLA-DPB1*0401/HLA-DPB1*0402 (107-119) (Fig. 6, *A*), and HLA-DR1 (110-131) (Fig. 6, *B*) were mapped on a computer model of the structure for the *Der p* I molecule. The epitopes are shown by displaying the side chains of component residues in a dark gray. It may be observed that the T-cell epitopes defined here are located on the exposed surface of this model of *Der p* I.

DISCUSSION

In this study we have investigated in an HDM-allergic individual the antigen and restriction specificity of the peripheral T-cell repertoire reactive with the group I allergen of *D. pteronyssinus* (*Der p* I). With peptide pools covering the entire sequence of *Der p* I and the group II allergen (*Der p* II) the antigen specificity of a T cell line reactive with *D. pteronyssinus* was examined. We observed that the major component of the HDM-reactive T-cell repertoire was directed toward residues 101-143 of *Der p* I and was cross-reactive with the closely related species of HDM, *D. farinae*. Analysis of fine antigen specificity of the T-cell response to this region was explored with T-cell clones isolated from the line and allowed three distinct T-cell epitopes to be mapped.

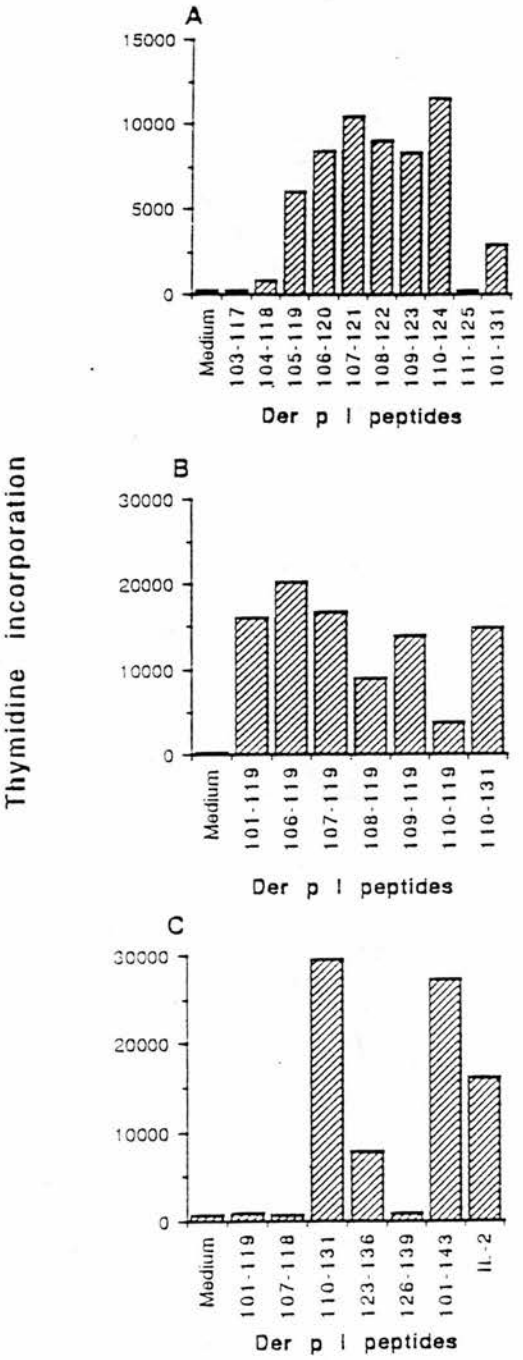

FIG. 3. Fine antigen specificity of *Der p* I–reactive T-cell clones. T cells of clones KS7.4 (A), KS2.12 (B), and KS2.15 (C) were stimulated with *Der p* I peptides in the presence of APCs. Proliferative responses at 1 μg/ml are shown.

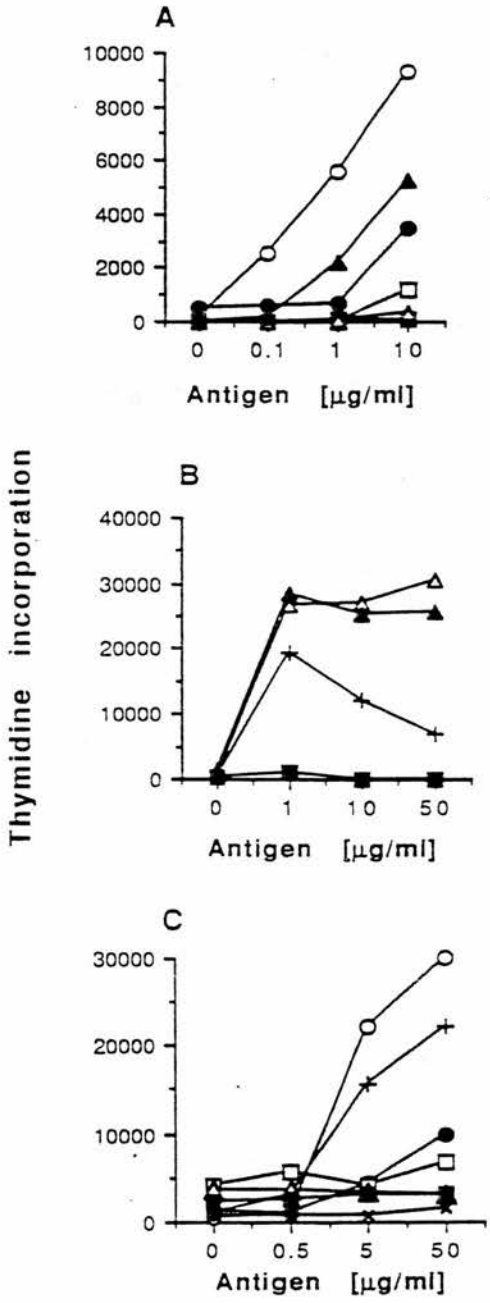

FIG. 4. HLA class II restriction specificity of *Der p* I–reactive T-cell clones. **A**, T cells of clone KS7.4 were stimulated with antigen in the presence of autologous EBV-transformed B cell line KSE (○) and WT24 (●) or with the mouse fibroblast lines: control DAP3 (□) and DAP3 transfected with DRB1*0101 (■), DPB1*0401 (△), and DPB1*0402 (▲). **B**, T cells of clone KS2.12 were stimulated with antigen in the presence of mouse fibroblasts transfected with DRB1*0101 (■), DPB1*0401 (△), DPB1*0402 (▲), and DPB1*0501 (+). **C**, T cells of clone KS2.15 were stimulated with antigen in the presence of EBV-transformed B cell lines KSE (○), WT24 (●), HOM2 (+), TISI (□), PGF (△), PMG075 (X), and YAR (■).

The T-cell clones KS2.12 and KS7.4 recognized two closely overlapping epitopes, 107-119 and 110-119, respectively; whereas the T-cell clone KS2.15 responded to residues 110-131. From sequence analysis it appears that there is 74% identity between *Der p* I and *Der f* I in the region 101-131 as opposed to an overall 81% identity for the two proteins; nevertheless, none of the T-cell clones analyzed were cross-reactive.[19] Amino acid substitutions at positions 108 (Ser to Ala), 110 (Arg to His), and 111 (Phe to Tyr), although conservative, appear to be important for T-cell recognition by the clones KS2.12 and KS7.4. Similarly, the unique specificity of KS2.15 for *Der p* I suggests that these residues and/or additionally those at residues 123-126 are critical. With the use of truncated recombinant proteins of *Der p* I, an independent study identified T-cell epitopes at residues 45-67, 94-104, and 117-143.[16]

Although in many instances T cells and B cells respond to distinct regions within a protein,[20] overlapping epitopes have been identified.[21] With regard to B-cell recognition of *Der p* I, analysis of specificity of serum IgE antibodies demonstrated that residues 60-80, 81-94, and 101-111 all contain B cell sites[22] and thus have only limited overlap with the T-cell epitopes. Mapping the T-cell epitopes defined here on a structural computer model of *Der p* I positions them on exposed surfaces of the molecule. Sequence analysis of *Der p* I protein (residues 94-143), which contain multiple T-cell epitopes restricted by different major histocompatibility complex molecules suggests that this region has low identity to homologous nonhuman cysteine proteases, such as cathepsin B, from which the model has been built. Differences in the sequence of *Der p* I and the corresponding self-proteins may account for the immunodominance of this region.

The ability of anti-HLA-DP, but not anti-HLA-DR framework antibodies, to inhibit antigen-dependent proliferation of the *Der p* I, 101-131–reactive T cell line indicated that HLA-DP class II molecules restrict T-cell recognition. Previous analysis of the restriction specificity of long-term HDM-reactive T cells has established that HLA-DRB1, HLA-DRB3, and HLA-DRB5 encoded gene products may present antigen.[15, 16, 23, 24] Using EBV-transformed B-cell lines and murine fibroblasts expressing alleles of HLA-

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                  10        20        30        40        50
                  •         •         •         •         •
DR B1 0101    LWQLKFECHFFNGTERVRLLERCIYNQEESVRFDSDVGEYRAVT

DP B1 0401    LFQGRQECYAFNGTQ  RFLERYIYNREEFARFDSDVGEFRAVT
DP B1 0402    ...............  ..............V.............
DP B1 0501    ...............  ..............LV............

                        60        70        80        90
                        •         •         •         •
DR B1 0101    ELGRPDAEYWNSQKDLLEQRRAAVDTYCRHNYGVGESFT

DP B1 0401    ELGRPAAEYWNSQKDILEEKRAVPDRMCRHNYELGGPMT
DP B1 0402    .....DE.....................................
DP B1 0501    .....E.....................................DEAV.
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FIG. 5. Primary amino acid sequence of the B1 domains of DRB1*0101, DPB1*0401, DPB1*0402, and DPB1*0501.

FIG. 6. The T-cell epitopes for (A) HLA-DPB1*0401/HLA-DPB1*0402/HLA-DPB1*0501 (107-119) and (B) HLA-DR1 (110-131) mapped on a computer model structure for the *Der p* I molecule. The epitopes are shown by displaying the side chains of component residues in dark gray.

DP, we observed that both the T-cell clones KS7.4 and KS2.12 responded to antigen in association with DPB1*0402. Although HLA-DP–restricted T-cell clones that are reactive with mycobacterial heat shock protein 65 or viral antigens have been reported,[25, 26] these HLA class II molecules appear to be less frequently used than HLA-DR. Antigen recognition by the T-cell clone KS7.4 was restricted by DPB1*0402 but not DPB1*0401 class II molecules, which differ at residues 38 (Ala to Val), 57 (Ala to Asp), 58 (Ala to Glu) (Fig. 5). On the basis of the crystalline structure of HLA-DRB1,[27] the residue at position 38 in HLA-DP would be located on the β-pleated sheets forming the floor of the cleft, whereas those at positions 57 and 58 would reside at one end of the α-helical wall of the antigen-combining site. Substitutions at any of these positions may affect peptide binding and T-cell recognition. The T-cell clone KS2.12 recognizes peptide in association with DPB1*0401, DPB1*0402, and DPB1*0501, despite amino acid differences at a number of positions (residues 37, 58, 86, 87, 88, and 89) in the β1 domain. Substitutions at positions 85 and 86 in DR class II molecules influence T-cell recognition for selected T-cell clones.[28] However, for the T-cell clone KS2.12, it appears that residue 86 is not critical for antigen recognition. The presence of a proline residue at position 88 is unique to certain HLA-DPB molecules and may cause a shortening of the β-chain helix, resulting in a conformational change at this end of the antigen-binding site. The substitution of this Pro by an Ala residue in HLA-DPB1*0501 would not have the same effect, which may account for the differential pattern of presentation by HLA-DPB*0501 class II molecules. The presence of polymorphisms at either end of the cleft and the ability of the peptide to be presented by the three HLA-DPB alleles suggest that the majority of binding interactions are formed in the center of the peptide. These results extend our earlier findings,[15] and together with recent genetic epidemiologic studies, which demonstrate a positive correlation between allergen-specific IgE and expression of certain HLA-DP alleles, highlight the potential importance of these class II molecules in atopic disease.[29, 30]

The third of the T-cell clones analyzed in this study, KS2.15, which is specific for the peptide residues 110-131, recognizes antigen presented by HLA-DRB1*0101 expressing APCs. HLA-DR restriction of *Der p* I peptides has been previously described, and in that study T-cell recognition of residues 45-67 and 117-143 of *Der p* I was restricted by HLA-DR7, and T-cell recognition of residues 94-104 was restricted by HLA-DR2, HLA-DR11 (DR5), and HLA-DR8.[16]

The development of subunit vaccines, targeted at allergen-specific CD4+ T cells, as potential immunotherapeutics for clinical desensitization requires the identification of immunodominant T-cell epitopes and the definition of their major histocompatibility complex class II restriction specificity. Our results together with other identified T-cell epitopes of *Der p* I[8, 16] suggest that residues 82-143 are an immunodominant region of *Der p* I, containing multiple T-cell epitopes restricted by different HLA class II molecules. Therefore any potential use of this region of *Der p* I as an immunotherapeutic for desensitization would not be limited to a restricted number of HLA class II specificities.

We thank Drs. Narayanaswamy Srinivasan, Noor A. Kalsheker, Christopher M. Topham, and John P. Overington for contributions in the modeling of *Der p* I; and Drs. Paul J. Travers and David S. Moss for contributions in the modeling of HLA-DP.

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Direct evidence for a functional role of HLA-DRB1 and -DRB3 gene products in the recognition of *Dermatophagoides* spp. (house dust mite) by helper T lymphocytes

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Key words: HLA class II, B3 gene products, T cell recognition, house dust mite, *Dermatophagoides* spp.

Abstract

The contribution of the HLA-DRB1, -B3, and -B5 gene products in the recognition of *Dermatophagoides* spp. (house dust mite) by helper T cells isolated from an atopic individual (HLA-DRw12, DR7; DRw52b) with perennial rhinitis was investigated. Using a panel of histocompatible and histoincompatible accessory cells, the restriction specificity obtained for a long term T cell suggested that a component of the dust mite reactive repertoire recognized antigen in association with DRB3 gene products. Oligonucleotide DNA typing of the presenting cell panel demonstrated a correlation between the DRw52b allele and T cell responsiveness. Murine fibroblasts expressing DRw52b, but not DRw52a or -c molecules, presented antigen to both the T cell line and cloned T cells (DE26) derived from the line, indicating that the supertypic specificity DRw52b was able to restrict recognition of dust mite antigens. Additional T cell clones (DE9 and DE41) also isolated from the line were restricted by the products of the B1 gene locus (DRw12B1) as determined by murine fibroblasts transfected with the appropriate HLA-DR genes. Clone DE9 was degenerate in its restriction specificity, also recognizing dust mite presented by accessory cells expressing the DR2 subtypes. Presentation by fibroblasts transfected with DRw12B1, DR2Dw2B5 genes and EBV-transformed B cell lines expressing DR2Dw21B1 and -B5 indicated that the functional site restricting recognition may be associated with residues 70 and 71 of the DRβ chain helical wall of the antigen combining site. Furthermore, we have recently demonstrated that both T cell clones DE9 and DE26 induce allergen dependent IgE synthesis *in vitro*. Thus these results demonstrate directly that the DRB1, -B3, and -B5 gene products are functional in the restriction of T cell recognition of dust mite antigens.

Introduction

Immune responses are initiated following the interaction of the antigen specific receptor of CD4+ T lymphocytes with molecular complexes formed between peptide fragments of antigen and MHC class II proteins (1–4). In man, MHC class II genes encode three major families of molecules, HLA-DR, -DQ, and -DP, all of which appear to function in the regulation of immune responsiveness (5–7). Furthermore, the DR locus on most haplotypes contains two expressed B genes, the products of which pair with the same DRα chain to give rise to two DR molecules, DR$\alpha\beta$I and DR$\alpha\beta$III (or DR$\alpha\beta$IV or DR$\alpha\beta$V), the major DR and the supertypic specificities respectively, both of which are polymorphic and able to restrict T cell antigen recognition (8–10).

Immune recognition of the aeroallergen *Dermatophagoides* spp. (house dust mite) induces immunoglobulin E (IgE) synthesis and symptomatic allergic disease in 10–15% of the population. Detailed analysis of the specificity and effector function of IgE in mite allergy is well documented (11–13). Although IgE

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Transmitting editor: T. Sasazuki

Received 17 October 1990, *accepted* 7 June 1990

synthesis is T cell dependent, only limited information is available on the antigen and MHC class II restriction specificity of the T cell repertoire activated in atopic individuals after exposure to house dust mite. Investigation of polyclonal responses to only the group I allergen of *Dermatophagoides pteronyssinus, Der p* I, indicated that T cells from atopic but not control non-atopic subjects proliferated to allergen (14). In addition, the results of experiments using anti-HLA class II antibodies to inhibit the antigen-dependent response of *D. pteronyssinus* reactive T cell clones and lines indicated that HLA-DR molecules restricted recognition of house dust mite but failed to identify the HLA-D region locus encoding the restriction elements used (15).

In previous studies we examined the HLA class II restriction of *D. farinae* reactive T cell clones isolated from the peripheral blood of an atopic individual using serological inhibition and allogeneic presenting cell panels (16,17). Complex patterns of restriction specificities were obtained suggesting that the product of locus DRAB3, in addition to the DRAB1 gene products, may function as restriction elements in T cell recognition. This prompted us to investigate the functional role of DRw52 class II molecules in the allergic response by correlating the patterns of reactivity of dust mite specific T cells with the results of DRB3 allele-specific hybridization with oligonucleotide probes in panel studies. Additionally, T cell clones capable of supporting allergen dependent IgE synthesis *in vitro* (18) were isolated from the T cell line and their restriction specificities examined using homozygous Epstein – Barr virus (EBV) transformed B cells and murine fibroblast transfectants expressing specific HLA-DR genes as antigen presenting cells (APC). The results of these experiments indicate that HLA-DRB1, -B3, and -B5 gene products are able to restrict the recognition of house dust mite and have allowed the mapping of functional sites on these HLA class II proteins.

Methods

Antigens

Lyophilized extracts of *D. farinae* were obtained from Pharmacia Ltd. Uppsala, Sweden.

Isolation of antigen reactive T cell line and clones

The isolation of the T cell line and clones used in these experiments has been described in detail elsewhere (16,17). Briefly, peripheral blood mononuclear leukocytes (PBMC; 2.5×10^5/ml) were stimulated with an optimal concentration of *D. farinae* [10^3 Biological Units (BU)/ml] for 7 days in RPMI 1640 (Flow Laboratories, Irvine, Ayrshire, UK) medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, and 5% v/v screened, heat-inactivated human A+ serum (National Blood Transfusion Centre, Edinburgh, UK). Lymphoblasts enriched on Ficoll-Paque (Pharmacia) were maintained as a long term line in the presence of irradiated (2500 rad) autologous PBMC, *D. farinae*, and interleukin 2 (IL-2, 10% v/v; Lymphocult T, Biotest Folex, Frankfurt, FRG), or cloned by limiting dilution from the line. For cloning, viable cells (0.3 cells/well) were plated in Microtest II trays together with irradiated autologous PBMC (5×10^5/ml), *D. farinae*, and IL-2. After 7 days, growing clones were transferred to 96-well flat-bottom microtitre trays and subsequently to 24-well trays. At each transfer the clones received filler cells, antigen, and IL-2. The clones and the line were maintained and expanded by the addition of IL-2 every 3 – 4 days and antigen together with filler cells every 7 days. Prior to their use in proliferation assays, the T cell clones and the line were rested for 6 – 8 days after the last addition of filler cells and antigen.

Antigen specificity of the T cell line and clones

The T cell line (DX) recognizes both major species of house dust mite (*D. farinae* and *D. pteronyssinus*) but the predominant specificity was for the group II allergen (*Der f* II; 12.5 kd) of *D. farinae* as determined using nitrocellulose immunoblots of fractionated antigen (16). T cell clones DE9 and DE26 responded to both dust mite species, with the determinant recognized by DE9 being further mapped to the group I allergens (26 kd). The T cell clone DE41 was only stimulated by *D. farinae* preparations.

Cell populations used as antigen presenting cells

Autologous and allogeneic PBMC, homozygous EBV transformed B cells, and murine fibroblasts transfected with HLA-D region genes were used as antigen presenting cells. The HLA-typed antigen presenting cell panel was generously provided by Dr D. Eckels, Blood Center of Southeastern Wisconsin, Milwaukee, WI. The cloning of the DR1Dw1, DRw2Dw2B1 (DRB1*1501), DR2Dw2B5, DRw12B1, DR7Dw17, and DRw52a, -b, and -c genes and their co-transfection with the DRA gene into the Ltk- fibroblast cell line (DAP3) has been described elsewhere (19 – 25).

T lymphocyte proliferation assays

T cells of the long term line (DX) and clones (DE9, DE26, and DE41; 5×10^4/ml) were cultured with antigen in the presence of irradiated HLA typed histocompatible and histoincompatible PBMC (1.25×10^5/ml), EBV transformed B cells (10^5/ml), or mitomycin C treated transfected murine fibroblasts (10^5/ml) in a total volume of 200 μl of complete medium in 96-well U-bottom plates. Assays in which fibroblasts were used as presenting cells were performed in 96-well flat-bottom plates. After 72 h of incubation, tritiated methyl thymidine (1 μCi/well, [^{3}H]TdR; Amersham International Inc., Amersham, UK) was added to cultures which were harvested onto glass fibre filters, 8 – 16 h later. Proliferation as correlated with [^{3}H]TdR incorporation was measured by liquid scintillation spectroscopy. The results are expressed as mean counts per minute (c.p.m.) + % error of the mean for triplicate cultures and were <20% in all experiments.

Mitomycin C treatment of murine fibroblasts

After trypsinization, cells were washed in serum free medium. Cells, up to 10^7/ml, were suspended in serum free medium and mitomycin C (Sigma) was added to a final concentration of 50 μg/ml. Cells were incubated at 37°C for 45 min, washed extensively in A+ supplemented medium, and used in proliferation assays (20).

Oligonucleotide DNA typing

DNA was prepared from the presenting cell panel and probed with oligonucleotides labelled with [γ-^{32}P]dATP as described previously (21). The oligonucleotide 52a (GGAGCT-GCGTAAGTCTGAG) probe is complementary to the nucleotide sequence residues 11 – 29 of DRw52a which encode for

amino acid residues 8 – 14 of the β1 domain of the DRw52a allele. Oligonucleotide 52b (GTTCCTGGAGAGACACTTCC) is complementary to the nucleotide sequence residues 62 – 81 of the DRw52b allele and oligonucleotide 52c (GTTCCTGGAGAGATACTTCC) is complementary to the nucleotide sequence 62 – 81 of the DRw52c allele (9). Nucleotide sequence residues 62 – 81 encode for amino acid residues 25 – 31 of the β1 domain of DRw52b and DRw52c.

Results

Identification of Dermatophagoides reactive T cell restricted by DRw52 associated determinants by oligonucleotide hybridization

The restriction specificity of a long term T cell line (DX) reactive with *D. farinae* was examined using a panel of histocompatible and histoincompatible PBMC, including RM (DRw12, DR7; DRw52), the autologous control, as accessory cells (Table 1). In each of the experiments examining the restriction specificity of the T cell line the complete panel of accessory cells was used and identical protocols were followed. The pattern of restriction was complex and revealed no clear correlation with the serologically defined B1 gene products. All of the accessory cells expressing DR7 were able to present *D. farinae* with the exception of 005G, which may reflect polymorphism in the DR7 allele. No proliferation was observed in the control cultures containing the T cell line and accessory cells in the absence of antigen. Although certain panel members had apparently identical specificity as determined serologically (150G and 814G; 857D and 826E), they differed in their efficiency as presenting cells. This may reflect microheterogeneity in the class II alleles that may not be detected unless the individual alleles are sequenced. Furthermore, a component of the long term T cell line recognized antigen presented by all the DRw11$^+$ and DRw12$^+$ accessory cells tested. Based on the sequence variation in the DR5 subtypes (DRw11 and DRw12), the pattern of proliferation suggests that the DRB3 gene product DRw52, as well as the DRB1 gene product, may also be restricting T cell recognition.

The supertypic specificity DRw52 is associated serologically with HLA-DR3, -5, -w13, and -w14. It was found by transfection studies to be encoded by the DRB3 locus (22) and sequence analysis has revealed at least three alleles at that locus (8). The restriction specificity of tetanus toxoid T cell clones has suggested a functional role for products of the DRB3 locus (10). To investigate further the possibility that DRB3 gene products were restricting T cell recognition of house dust mite antigens, the panel of presenting cells was typed using oligonucleotide probes specific for the DRw52a, -b, and -c alleles. The results demonstrated a complete correlation between the DRw52b oligonucleotide hybridization pattern in those cells expressing DRw52 (not donors 305G and 239G) and the induction of antigen dependent proliferation (Table 1). In contrast, the T cells failed to recognize *D. farinae* presented by accessory cells expressing DRw52a and -c.

Antigen presentation by murine fibroblasts expressing DRw52b class II molecules

To confirm the functional role of DRw52 in the recognition of *D. farinae*, murine fibroblasts transfected with DRw52a, -b, or -c genes were used as antigen presenting cells. Only those fibroblasts expressing DRw52b, but not DRw52a and -c, were able to present antigen in a dose dependent manner to T cells of the long term line DX (Fig. 1). Comparison of the primary sequence reveals that amino acid substitutions at positions 30, 37, 38, 51, 57, and 60 of the β chain distinguish between DRw52b and the other two subtypes, DRw52a and -c (Table 2).

Table 1. Restriction specificity of the house dust mite reactive T cell line

Presenting cell	Haplotype			Response (Δ c.p.m.)
	DR	DRw		
		52	53	
RM	7,w12	b	–	28.501
088G	1,5	b	–	2484
150G	4,w12	b	+	11.855
814G	4,w12	b	+	2694
056G	5,-	b/b	–	6802
857D	5,6	b	–	18.074
826E	5,6	b	–	6424
005G	1,7	–	+	271
305G	2,7	–	+	15.320
239G	7,9	–	+	4499
161E	2,9	–	+	202
855E	2,4	–	+	173
001C	2,-	–	–	798
1009	3,8	a	–	285
004C	3,-	a	–	877
804E	3,6	a/c	–	482

The long term T cell line was stimulated with *D. farinae* in the presence of a panel of histocompatible and histoincompatible irradiated PBMC as a source of APCs. Background responses to APCs in the absence of antigen have been subtracted in each case (< 1000 c.p.m.). Culture conditions and proliferation were determined as described in Methods.

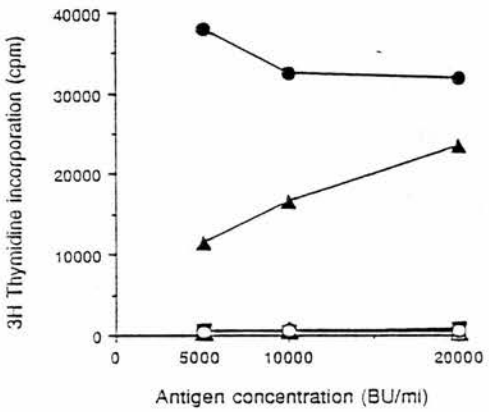

Fig. 1. Proliferative response of the T cell line DX to *D. farinae* presented by murine fibroblasts expressing the DRw52 alleles. T cells (10^5/ml) were cultured with *D. farinae* ($5 – 20 \times 10^3$ BU/ml) in the presence of autologous PBMC (1.25×10^5/ml; ●) or mitomycin C-treated murine fibroblasts (10^5/ml) expressing DRw52a (□), DRw52b (▲), DRw52c (△), DR1Dw1 (■), and Ltk$^-$ cells (○). Background responses of T cells to accessory cells in the absence of antigen were < 500 c.p.m. Culture conditions and proliferation were determined as described in Methods.

No proliferation was obtained when antigen was presented by control fibroblasts expressing DR1. The failure of L cell transfectants to present house dust mite components in the experiments described in this paper could not be attributed to their lack of functional activity as they supported antigen presentation in other models of T cell antigen recognition (20,24, personal communications). Stimulation of T cells by the house dust mite was markedly greater in the presence of autologous PBMC. The absence of accessory molecule interactions and the potential for differential processing by murine fibroblasts may account for the less efficient antigen presentation.

In order to examine the relative contribution of the DRB1 and B3 gene products in the recognition of house dust mite antigens the T cell line was cloned by limiting dilution. The patterns of proliferation obtained with T cell clones DD11, DE12, and DE26 isolated in this way were similar to that observed for the T cell line as regards their response to antigen presented by DR5+ (DRw11+ and DRw12+) cells (17). Consequently, the restriction specificity of T cells of clone DE26 was examined using DRw52 transfected murine fibroblasts. Antigen dependent proliferation was obtained only in the presence of fibroblasts expressing DRw52b (Fig. 2). Neither the DRw52a nor the DRw52c transfectants were able to present antigen to DE26. Similarly, in control cultures containing DR1+, DR7+, and Ltk- fibroblasts no proliferation was observed. Thus allergen reactive T cells restricted by the DRAB3 gene product (DRw52b) could be identified at both the oligoclonal and monoclonal levels.

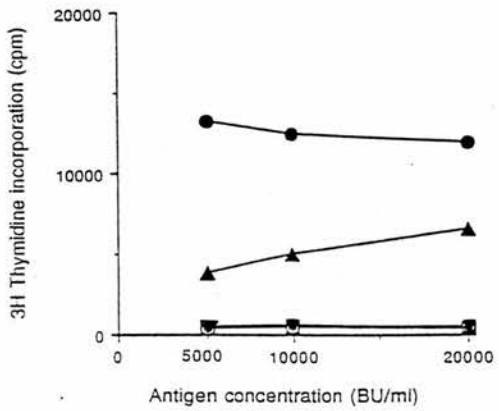

Fig. 2. Recognition of *D. farinae* by T cell clone DE26 is restricted by the DRw52b specificity. Cloned T cells (10^5/ml) were cultured with *D. farinae* (5 – 20 × 10^3 BU/ml) in the presence of autologous PBMC (1.25 × 10^5/ml; ●) or mitomycin C-treated murine fibroblasts (10^5/ml) expressing DRw52a (□), DRw52b (▲), DRw52c (△), DR1Dw1 (■), DR7Dw17 (▼), and Ltk- cells (○). Background responses of T cells to accessory cells in the absence of antigen were <350 c.p.m. Culture conditions and proliferation were determined as described in Methods.

DRAB1- and DRAB5-restricted T cell recognition of Dermatophagoides

From the pattern of T cell proliferation of the long term line observed using the allogeneic presenting cell panel (Table 1) and from the results of previous experiments (17), it appeared that DRAB1 gene products also contributed to the recognition of house dust mite allergen by T cells of this individual (Table 1). The restriction specificities of the T cell clones DE9 and DE41, derived from the T cell line, were examined in detail.

T cells of clone DE9 were stimulated by antigen in association with some DR2+, DR5+, and DR8+ accessory cells (17).

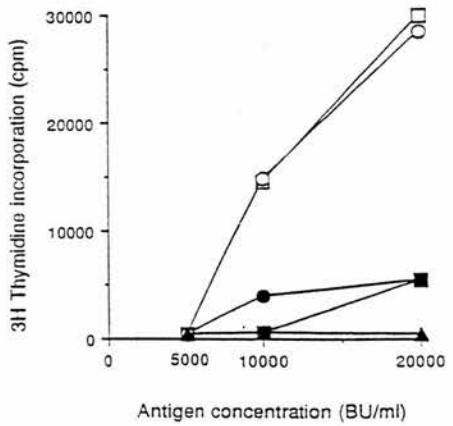

Fig. 3. EBV transformed B cells expressing DR2 or DR5 class II proteins restrict the recognition of *D. farinae* by the T cell clone DE9. Cloned T cells of DE9 (10^5/ml) were cultured with *D. farinae* (5 – 20 × 10^3 BU/ml) in the presence of autologous PBMC (1.25 × 10^5/ml; ●), or EBV-transformed B cells (10^5/ml) expressing DRw2Dw2 (□), DR2Dw21 (■), DRw12 (○), or DR7Dw17(▲). Background responses of T cells to accessory cells in the absence of antigen were <1400 c.p.m. Culture conditions and proliferation were determined as described in Methods.

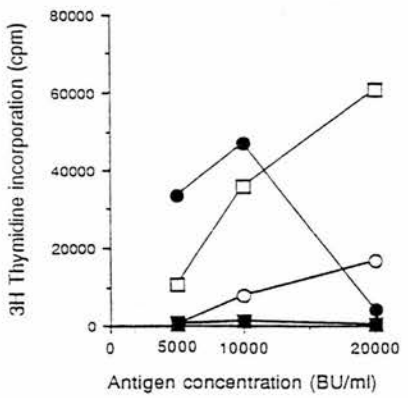

Fig. 4. Mapping the fine restriction specificity of T cell clone DE9 with murine fibroblasts expressing the DRw12 and DR2Dw2 specificities as presenting cells. Cloned T cells of DE9 (10^5/ml) were cultured with *D. farinae* (5 – 20 × 10^3 BU/ml) in the presence of autologous PBMC (1.25 × 10^5/ml; ●) or mitomycin C-treated murine fibroblasts (10^5/ml) expressing DRw12B1 (□), DR2Dw2B5 (○), DR2Dw2B1 (■), and DR1Dw1 (▲) gene products. Background responses of T cells to accessory cells in the absence of antigen were <940 c.p.m. Culture conditions and proliferation were determined as described in Methods.

Homozygous EBV-transformed B cells expressing DR2Dw2 or DR2Dw21 molecules were able to present mite antigens to the T cell clone (Fig. 3). However, their capacity to present antigen was quantitatively different, with DR2Dw2 EBV B cells being markedly more effective. Nevertheless, presentation of the DR2Dw21+ B cells was always >4-fold that of the background accessory cells alone and antigen presented by class II molecules of unrelated specificity. Similarly, the parental allele DRw12+ but not the control DR7+ EBV B cells induced antigen dependent proliferation of T cell clone DE9 (Fig. 3).

To determine the contributions of DRB1 and DRB3 gene products to the presentation of antigen by DRw12, DR2Dw2, and DR2Dw21 expressing EBV B cell lines, murine fibroblasts transfected with the appropriate HLA class II genes were used as presenting cells. Fibroblasts expressing the DRw12B1 or DR2Dw2B5 gene products were able to present antigen to clone DE9 in a dose dependent manner (Fig. 4). In contrast, the DR2Dw2B1 gene expressed in fibroblasts failed to induce T cell proliferation. In the absence of antigen or with control DR1Dw1+ transfectants no activation of the T cells was observed. From the results of previous experiments T cell clone DE41 appeared to be restricted by DRw12+ (DR5) accessory cells (17). Indeed, murine fibroblasts transfected with the DRw12B1 gene were able to present *Dermatophagoides* to the T cell clone DE41 (Fig. 5). However, the efficiency of presentation by these cells was reduced as compared with autologous PBMC.

Discussion

In this paper the HLA class II restriction specificity of T cell recognition of the house dust mite, *Dermatophagoides* spp., is examined using T cell clones isolated from the peripheral blood of an atopic individual with perennial rhinitis (16,17). To investigate the heterogeneity of the HLA class II alleles regulating the responsivenss to house dust mite allergens a panel of HLA-DR histocompatible and histoincompatible PBMC was used to present antigen to an oligoclonal T cell population. The T cell line responded to both major species of the house dust mite, although the dominant specificity was for component(s) of the group II allergen of *D. farinae*. Although T cell recognition was HLA class II restricted, the pattern of the proliferative responses did not correspond to serologically defined DRB1 gene products. However, correlation between the functional studies and oligonucleotide typing of the presenting cell panel strongly suggest that the B3 gene products were able to restrict recognition of components of house dust mite. Although DRw52a restricted T cell recognition of tetanus toxoid has been reported, unlike the response to dust mite (17), the major component of the tetanus toxoid reactive repertoire is not restricted by B3 gene products (10). Furthermore, using murine fibroblasts expressing each of the three alleles of DRw52 (10) as presenting cells, the restriction determinants were assigned to the DRw52b molecule. Similarly, cloned T cells reactive with a shared determinant in both species of dust mite (DE26) were isolated from the T cell line and showed identical restriction specificity for DRw52b expressed on murine fibroblasts. These findings demonstrate directly that the product of locus DRB3 is functional in antigen presentation and that it contributes to the recognition of house dust mite allergens. The magnitude of the proliferative response induced in the T cell line when *D. farinae* was presented by DRw52b+ fibroblasts indicated that this was a major restriction element. This result is somewhat surprising for, although DR proteins, as opposed to DQ and DP (26), are the main restriction elements used in T cell recognition, this can be mapped to the B1 gene products in most cases. Quantitation of mRNA for class II proteins suggests that the B3 gene products are expressed at 2- to 5-fold lower levels than the B1 gene products (27). Thus, the dominance of the DRw52 allele as a restriction element in the recognition of the house dust mite by this individual is independent of the differential expression of the B1 and B3 gene products and may reflect differences in the binding of house dust mite peptides or in the T cell repertoire activated in the allergic immune response, with a very different pattern of allelic diversity observed among the DRB3 alleles than among the DRB1 alleles (10). As the specific T cell response of other house dust mite allergic individuals is inhibitable by an antibody reactive with DRw52b, this indicates that the contribution of the B3 gene products in the recognition of *Dermatophagoides* is not limited. The immunodominance of allele 52b in the case of the house dust mite is also of interest in view of the wide distribution of that allele.

From comparison of the primary amino acid sequence of the first domain of the β chain of the DRw52 alleles, differences in positions 30, 37, 38, 50, 57, and 60 distinguish DRw52b from the other two DRw52 alleles (Table 2). Based on the three dimensional structure of HLA-A2 (28) and the sequence homologies between class I and class II histocompatibility proteins, a hypothetical model of the antigen combining site of MHC class II molecules has been generated (29). From their proposed location in the combining site, the amino acids at positions 30, 37, 57, and 60 may influence T cell recognition. Residues at positions 30 and 37 are located on the central strands

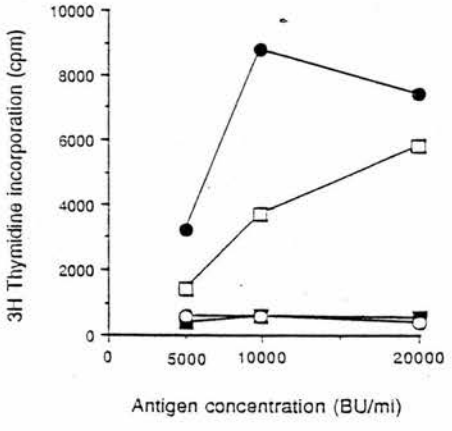

Fig. 5. Recognition of *D. farinae* by T cell clone DE41 is restricted by the DRw12 class II specificity. T cells of clone DE41 (10^5/ml) were cultured with *D. farinae* ($5-20 \times 10^3$ BU/ml) in the presence of autologous PBMC (1.25×10^5/ml; ●) or mitomycin C-treated murine fibroblasts (10^5/ml) expressing DRw12B1 (□), DR1Dw1 (■), and Ltk− cells (○). Background responses of T cells to accessory cells in the absence of antigen were <410 c.p.m. Culture conditions and proliferation were determined as described in Methods.

Table 2. The primary amino acid sequence of the B1 domains of supertypic specificities DRw52a, -b, and -c

	10	20	30	40
DR cons	P R F L E Q x K S E C H F F N G T E R V R x L x R x F H N Q E E x V R F D S D V G E Y R A			
DRw52a	- - - - - L R - - - - - - - - - - - - - - - - Y - D - Y - - - - - - F L - - - - - - - - - - -			
DRw52b	- - - - - L L - - - - - - - - - - - - - - - - F - E - H - - - - - - Y A - - - - - - - - - - -			
DRw52c	- - - - - L L - - - - - - - - - - - - - - - - F - E - Y - - - - - - F V - - - - - - - - - - -			

	50	60	70	80	90
DR cons	V x E L G R P x A E x W N S Q K D L L E Q K R G A V D N Y C R H N Y G V x E S F T V Q R R				
DRw52a	- T - - - - - V - - S - - - - - - - - - - - - - - - - R - - - - - - - - - - G - - - - - - - -				
DRw52b	- R - - - - - D - - Y - - - - - - - - - - - - - - - - Q - - - - - - - - - - V - - - - - - - -				
DRw52c	- T - - - - - V - - S - - - - - - - - - - - - - - - - Q - - - - - - - - - - V - - - - - - - -				

Table 3. The primary amino acid sequence of the B1 domains of DRw12B1, DR2Dw2B1, -Dw2B5, -Dw21B1, and -Dw21B5 MHC class II proteins

	10	20	30	40
DR cons	P R F L E Q x K x E C H F F N G T E R V R F L x R Y F Y N Q E E Y V R F D S D V G E Y R A			
DRw12B1	- - - - - Y S T G - - Y - - - - - - - - - - L - E - H - H - - - - L L - - - - - - - - - F - -			
DR2Dw2B5	- - - - Q - D - Y - - - - - - - - - - - - - - - - H - D I - - - - - D L - - - - - - - - - - -			
DR2Dw2B1	- - - - W - P - R - - - - - - - - - - - - - - - - D - - - - - - - - S V - - - - - - - - F - -			
DR2Dw21B5	- C - - Q - D - Y - - - - - - - - - - - - - - - - H - G I - - - - - N V - - - - - - - - - - -			
DR2Dw21B1	- - - - W - P - R - - - - - - - - - - - - - - - - D - - - - - - - - S V - - - - - - - - - - -			

	50	60	70	80	90
DR cons	V T E L G R P D A E Y W N S Q K D L L E Q x R A A V D T Y C R H N Y G V G E S F T V Q R R				
DRw12B1	- - - - - - - - V - - S - - - - - - - I - - D R - - - - - - - - - - - - - - - A V - - - - - - - -				
DR2Dw2B5	- - - - - - - - - - - - - - - - - - F - - D R -				
DR2Dw2B1	- - - - - - - - - - - - - - - - - - I - - - A - - - - - - - - - - - - - - - - V - - - - - - - -				
DR2Dw21B5	- - - - - - - - - - - - - - - - - - I - - - A - - - - - - - - - - - - - - - - A V - - - - - - - -				
DR2Dw21B1	- - - - - - - - - - - - - - - - - - F - - D R -				

of the β pleated sheets forming the floor of the combining site, whereas positions 57 and 60 would be located at one end of the α helical wall of the cleft. All four residues are remarkably clustered on the DR molecule. Substitutions at any of these positions may affect antigen binding and T cell recognition. Similarly, the residue at position 38 may influence the conformation of the α helical wall and thus restrict the ability of certain peptides to occupy the antigen combining site. By site directed mutagenesis it has been demonstrated that a single amino acid substitution at position 29 in the floor of the antigen combining site could determine T cell recognition of pigeon cytochrome *c* (30). This would imply that amino acid variation at position 30 may be critical for T cell recognition of house dust mite peptides restricted by the DRw52 molecule. Likewise, residue differences at position 57 alone in the DQβ chain have been reported to influence the susceptibility to insulin dependent diabetes (31). To extend this analysis on the contribution of the polymorphic residues in DRw52 on T cell recognition of dust mite, defined T cell epitopes are required. However, this is hampered by a lack of structural information on the components of mites, with only the group I allergens of *D. pteronyssinus* having been cloned and sequenced. Nevertheless, the ability of unfractionated house dust mite allergen to competitively inhibit the binding of peptides to fibroblasts expressing DRw52 supports the role of the B3 gene products as the major restriction elements in the recognition of *Dermatophagoides* (R. E. O'Hehir and J. R. Lamb, unpublished results).

Population analysis of the immunogenetic basis of atopy has indicated associations between the HLA-DR phenotype and the induction of specific IgE for several purified allergens (32 – 34). The isolation of T cell clones from the long term line also allowed the functional sites on the B1 gene products capable of restricting recognition of house dust mite determinants to be mapped. The restriction specificity of the T cell clone DE9, which recognizes an invariant sequence in the group I allergens of the house dust mite, was degenerate in that antigen was recognized in association with the parental allele DRw12 as well as DR2Dw2 and DR2Dw21. This is in contrast to the *D. farinae* reactive T

cell clone DE41, which is restricted by DRw12 but shows no degeneracy on the DR2Dw2 or DR2Dw21 proteins. Interestingly, the presence of serum IgE specific for the pollen antigens, *Amb a* V and *Amb a* VI, are DR2Dw2 and DR5 (DRw11) associated, respectively (32,33). Amino acid sequence comparison of the β1 domains of DRw12B1 (DRB1*1201) and DR2Dw2B5 reveals extensive differences in the N-terminal half of the domains (Table 3), the region predicted to compose the floor of the antigen combining site (29). In contrast, the C-terminal half of these domains, predicted to form the α helix running across the floor of anti-parallel strands, is similar for these two DR molecules, with identity at positions 70 and 71. The DR2Dw2B1 and DR2Dw21B5 α helical sequences differ from DRw12B1 and DR2Dw2B1 at these positions; however, the sequence of the DR2Dw21B1 gene product is identical. These observations imply that the HLA class II restriction of this T cell clone is determined by allelically polymorphic residues in the α helix of the β1 domain, irrespective of sequence differences in the floor (35). Similar patterns of MHC restriction have been described for the influenza haemagglutinin-specific, DR1Dw1-restricted T cell clone, HA1.7 (36), and for multiple murine T cell clones (1,35). Another implication of the results of these experiments is that, although the B1 gene product is the restriction element on the DRw12 and DR2Dw21 haplotypes, it is probable that the B5 gene product is used in the case of the DR2Dw2-expressing cells. This can be inferred from the sequence identity of these molecules at positions 70 and 71. Similar flexibility of MHC restriction may account for the DR3/DR5 association of the allergic immune response to the rye allergen, *Lol p* III (34), since these alleles have different sequences but share the same B3 gene sequence. This may be the molecular basis for DR3/DR5 restricted recognition of *Lol p* III as opposed to conserved residues (amino acids 9 – 13) in the β strands of the floor of the antigen combining site (34).

The findings reported here demonstrate the heterogeneity of MHC class II restriction of T cell recognition of house dust mite antigens within an individual in terms of the use of the B1 and B3 gene products. Furthermore, both clones DE9 and DE26, although restricted by B1 and B3 gene products respectively, are able to provide help for IgE synthesis in an allergen dependent *in vitro* B cell assay (17,18). It is surprising that the major component of the T cell repertoire is restricted by the B3 gene products in view of their reduced expression relative to the B1 locus products (27). Contrary to the B1 gene products, where the polymorphism is restricted to three variable domains, in the DRw52 alleles sequence differences are scattered throughout the β1 domain (Table 2). This may regulate T cell recognition qualitatively by restricting the diversity of peptides capable of occupying the antigen combining site (37), in this case DRw52b, and the subsequent activation of T cells which requires co-recognition of MHC class II and peptide. The immunological advantage of B1, B3, and B5 gene products functioning as restriction elements may be to increase the diversity of the T cell repertoire activated in response to the house dust mite, although in some individuals sensitized with allergen the population of T cells activated may induce an aberrant immune response.

The heterogeneity of the antigen and restriction specificity of T cell recognition of the house dust mite is of practical importance for the development of therapy based on immunological intervention, such as the induction of T cell tolerance or competitive inhibition of MHC class II proteins (38).

Acknowledgements

The authors wish to thank Professors A. B. Kay and J. R. Batchelor for helpful discussions. J. R. Lamb is a member of the External Scientific Staff of the Medical Research Council and R. E. O'Hehir is supported by a Wellcome Senior Research Fellowship in Clinical Science.

Abbreviations

APC	antigen presenting cells
BU	biological units
EBV	Epstein – Barr virus
IL-2	interleukin 2
PBMC	peripheral blood mononuclear leukocytes
TdR	methyl thymidine

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International Immunology, Vol. 5, No. 12, pp. 1589–1597

Clonal analysis of the atopic immune response to the group 2 allergen of *Dermatophagoides* spp.: identification of HLA-DR and -DQ restricted T cell epitopes

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Key words: DR restriction, DQ restriction, HLA class II, house dust mite, T cell recognition

Abstract

The group 2 allergens of *Dermatophagoides* spp. (house dust mite, HDM) are a major immunological target for IgE antibodies in the allergic immune response of HDM atopic individuals. In this report the heterogeneity of the T cell repertoire reactive with the group 2 allergen of *Dermatophagoides pteronyssinus* (Der p 2) of a HDM allergic individual was investigated using overlapping synthetic peptides. By clonal analysis four distinct T cell epitopes were identified, located within residues 16–31, 22–40, 82–100, and 111–129. The importance of these epitopes was confirmed by investigation of the peripheral T cell repertoire, with the polyclonal T cell response to Der p 2 failing to show marked variations in epitope specificity over time. Serological inhibition studies and the use of Epstein–Barr virus transformed B cell lines characterized for their expression of HLA-D region gene products demonstrated that recognition of peptides 16–31 and 111–129 was restricted by HLA-DQ (DQB1*0301), whereas peptide 82–100 is recognized in association with HLA-DR (DRB1*1101). Peptide 22–40 was presented by both HLA-DRB1*1101 and -DQB1*0301 class II molecules. The potential application of these findings lies in the design of peptide-based immunotherapeutics for the management of HDM allergic disease.

Introduction

The exposure of genetically susceptible individuals, ~10–15% of the population, to environmental allergens including the proteins of house dust mite (HDM) results in clinical symptoms that range from extrinsic asthma and allergic rhinitis to atopic dermatitis (1). The inflammatory responses that cause these clinical symptoms are due to the synthesis of allergen specific IgE antibodies and the activation of polymorphonuclear granulocytes (2,3). Initially, interest focused on the specificity of IgE antibody responses and, although multiple proteins have been identified in HDM that bind IgE from the sera of HDM allergic individuals (4), the major component of the HDM reactive B cell repertoire is directed towards the group 1 and 2 proteins and is cross-reactive to both species of HDM, *Dermatophagoides pteronyssinus* and *D. farinae* (5,6). Furthermore, it appears from several studies that between 40 and 88% of the IgE antibody response is specific for the group 2 allergens (Der p 2 and Der f 2), and illustrates their importance as target antigens in the allergic immune response to HDM (7).

It is well established that the induction of the allergic inflammatory response, including allergen specific IgE synthesis and the growth, differentiation and activation of non-specific granulocytes depends on the activity of $CD4^+$ T cells (2,3,8).

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Transmitting editor: A. Cooke

Received 14 May 1993, *accepted* 13 September 1993

$CD4^+$ T cell activation requires the combined effects of co-stimulatory signals and occupancy of specific TCR by peptide fragments of antigen complexed with class II MHC molecules (9–11). In order to understand the pathogenesis of allergic inflammatory responses and to develop new approaches to immunotherapy, information on both the antigen and MHC class II restriction specificity of T cell recognition of the major proteins of HDM is required. With the primary amino acid sequences of the group 2 allergen available from the nucleotide sequence of cloned cDNA (12,13), it is possible to identify the immunodominant epitopes in this protein that are recognized by the T cell repertoire of HDM allergic individuals (14).

Genetic epidemiological studies have failed to establish a clear association between the expression of a particular HLA phenotype and allergic responsiveness to HDM (15,16), and therefore much of the information available on those HLA class II molecules that contribute to HDM responsiveness is based on characterization of the restriction specificity of T cell clones (17,18). These studies have demonstrated that HLA-DRB1, -DRB3, -DRB5, and -DP encoded gene products all may function as restriction elements in T cell recognition of HDM allergens (19–21). However, at present detailed analysis of the restriction specificity of T cell recognition of Der p 2 at the epitope level is incomplete. This prompted us to investigate by clonal analysis the heterogeneity of T cell responses to Der p 2. With overlapping peptides we have identified four distinct T cell epitopes at residues 16–31, 22–40, 82–100, and 111–129. Furthermore, from serological inhibition studies and the patterns of proliferation observed when specific peptide was presented by antigen presenting cells (APC) expressing different HLA class II molecules, it appeared that recognition of peptides 16–31 and 111–129 was restricted by HLA-DQ (DQB1*0301), whereas peptide 82–100 was recognized in association with HLA-DR (DRB1*1101). Peptide 22–40 was presented by both HLA-DRB1*1101 and -DQB1*0301 class II molecules. The analysis of the peptide sequences suggests that allele specific anchor residues are present determining binding to DQB1*0301.

Methods

Antigens

Overlapping peptides derived from the nucleotide sequence of Der p 2 were synthesized in our department as previously described (22), or purchased from Chiron Mimotopes (Melbourne, Australia). The primary amino acid sequence of Der p 2 is shown in Fig. 1.

Antibodies

The monoclonal anti-HLA-DR (L243) (23), anti-HLA-DP (B7/21) (24), and anti-HLA-DQ (SPV-L3) (25) antibodies were isolated from the culture supernatants of the respective hybridoma cell lines and purified using Protein A–Sepharose.

Isolation of Der p 2 reactive T cell clones

T cell clones reactive with Der p 2 were isolated from a HDM allergic individual [AC; haplotype DR7, DR11(5), DQ2, DQ7(3), DPB1*0101/*0402] by limiting dilution cloning as described previously (14,17). Briefly, peripheral blood mononuclear cells (PBMC; 10^6/ml) were stimulated with an optimal concentration of Der p 2 (20 μg/ml) for 7 days in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 IU/ml penicillin/streptomycin (Gibco, Life Technologies, Paisley, UK) and 5% screened, inactivated human A^+ serum (National Blood Transfusion Service, Edgware, UK). A long-term line of the donor was established with lymphoblasts enriched on Ficoll-Paque (Pharmacia, Milton Keynes, UK), in the presence of irradiated autologous PBMC (5×10^5/ml; 2500 rad), Der p 2 (20 μg/ml)

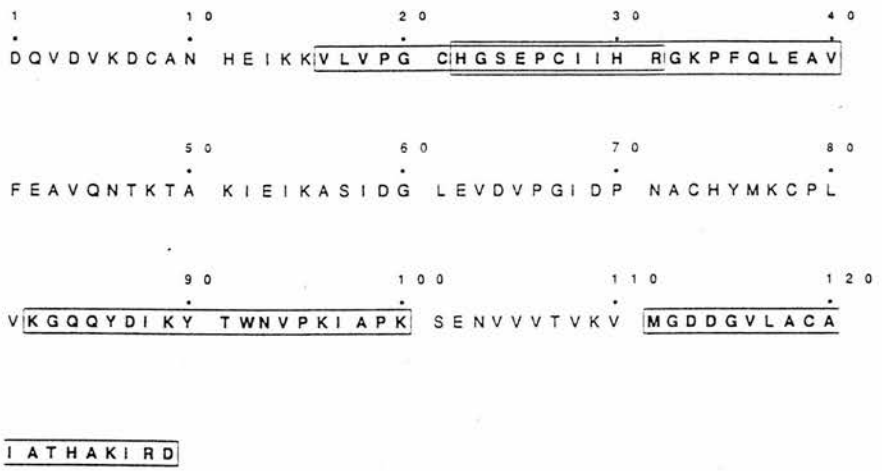

Fig. 1. Primary amino acid sequence of the Der p 2 molecule (boxed residues refer to the defined epitopes).

and IL-2 (IL-2, 10% v/v; Lymphocult T, Biotest Folex, Frankfurt, Germany). Viable cells (0.3 cells/well) were cloned by limiting dilution in supplemented medium and plated in 96-well flat bottom plates (Nunc, Gibco Life Sciences, Paisley, UK) together with an irradiated autologous Epstein – Barr virus-transformed B cell line (EBV-B cell line) (1×10^5/ml; 5000 rad), allogeneic PBMC from two donors (1×10^6/ml total; 2500 rad), Der p 2 (20 μg/ml), and Leuco A (1 μg/ml) (Pharmacia, Milton Keynes, UK). After 7 days, growing clones were transferred to 24-well plates. The clones were maintained with IL-2 every 3 – 4 days and irradiated autologous PBMC and Der p 2 every 7 days. Clones were rested for 7 – 8 days after the last addition of antigen and antigen presenting cells before experimental procedures.

Proliferation assays

Polyclonal T cell assays. Unfractionated PBMC (1×10^6/ml) were cultured with Der p 2 peptides (10 and 30 μg/ml). On day 7, T cell proliferation was measured by pulsing the cultures with

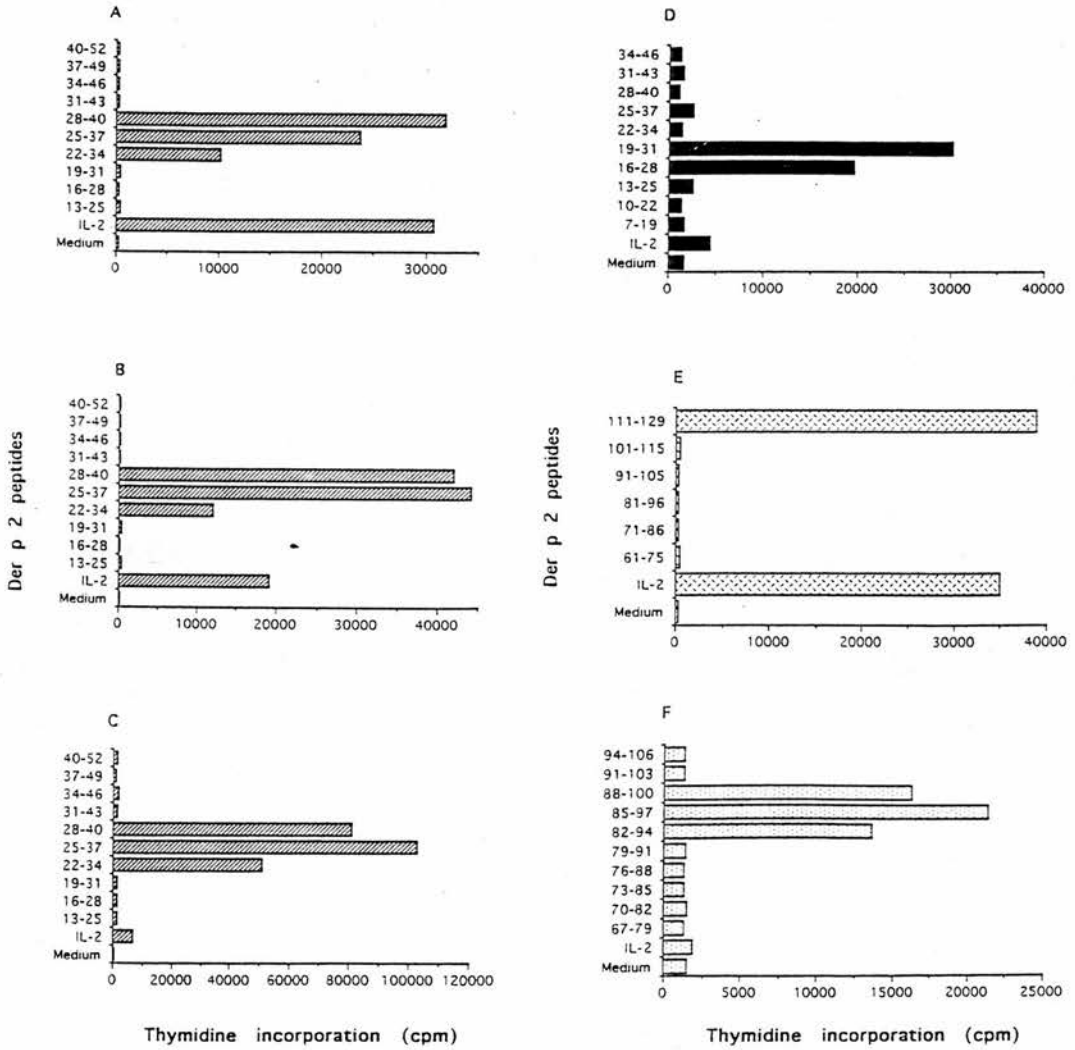

Fig. 2. Epitope specificity of Der p 2 specific T cell clones. A, AC1; B, AC2; C, AC3; D, AC5; E, AC28; and F, AC29. T cell clones were stimulated with overlapping peptides of Der p 2 at 1 and 10 μg/ml in the presence of APCs (irradiated autologous EBV-B cells). The maximal proliferative response obtained for each peptide is shown. Control responses to IL-2 or medium alone were also determined.

tritiated methyl thymidine (1 μCi/well, [^{3}H]TdR; Amersham International, Amersham, UK) and the cultures harvested 8 – 16 h later. Proliferation as correlated with [^{3}H]TdR incorporation was measured by liquid scintillation spectroscopy. The results are expressed as mean c.p.m. for triplicate cultures. The standard error of the mean for all experiments was <20%.

Clonal T cell responses. Peptides (0.1 – 50.0 μg/ml) were presented to cloned T cells (10^5 cells/ml) with irradiated autologous PBMC (2500 rad), autologous EBV-B cells or HLA typed EBV-B cells, histocompatible or histoincompatible at the D region loci (5000 rad), as a source of APCs. In serological inhibition assays, antibodies were added over a concentration range at the initiation of cultures. After 60 h incubation the cultures were pulsed with [^{3}H]TdR (1 μCi/well) and harvested as described for the polyclonal proliferation assays. The results are expressed as mean c.p.m. for triplicate cultures. The standard error of the mean for all experiments was <20%.

Results

Epitope specificity of T cell clones induced with Der p 2

In order to investigate the heterogeneity of the peripheral T cell repertoire reactive with Der p 2, a panel of T cell clones was isolated and their epitope specificity mapped using a set of overlapping peptides derived from the primary amino acid sequence of Der p 2 (Fig. 1). Each peptide was tested at 1 and 10 μg/ml but only the maximal proliferation observed at either concentration is presented (Fig. 2). The T cell clones AC1, AC2, and AC3 all responded to three overlapping peptides (residues 22 – 34, 25 – 37, and 28 – 40) spanning the sequence 22 – 40 (Fig. 2A – C). The optimal responses for AC1 and AC2 were obtained using 1 μg/ml of peptide (residues 25 – 37 and 28 – 40) and 10 μg/ml (residues 22 – 34). For AC3, 10 μg/ml was optimal for all three stimulatory peptides. The remaining clones, AC5, AC28, and AC29, were all maximally stimulated using 10 μg/ml. However, the pattern and magnitude of the responses to the nested set of peptides were variable with peptide 22 – 34 being the least potent at inducing proliferation of the T cell clones. T cell clones AC1 (Fig. 2A) and AC3 (Fig. 2C) proliferated more markedly to residues 28 – 40 and 25 – 37 respectively, whereas AC2 (Fig. 2B) responded equally to both peptides. This implies that these three clones recognize epitopes within residues 22 – 40, but, there may be slight differences in the fine epitope specificity. The dominance of the response to peptide Der p 2-(28 – 40) by AC1 implies that this clone may recognize an epitope towards the C-terminus of this region. On the other hand, AC3 may recognize an epitope located more towards the N-terminus as peptide Der p 2-(25 – 37) induces greater proliferation. However, the similar response of AC2 to both peptides may imply that there is a third epitope within residues 22 – 40. Peptides 19 – 31 and 16 – 28, but none of the flanking sequences activated T cell clone AC5 (Fig. 2D), whereas T cell clone AC28 responded only to the C-terminal region peptide, amino acids 111 – 129 (Fig. 2E). Peptide 85 – 97 induced maximal proliferation of T cell clone AC29 (Fig. 2F), and responses were also observed to the flanking sequences 82 – 94 and 88 – 100. Collectively, these results demonstrate the presence of at least four distinct T cell epitopes that are recognized by the peripheral T cell repertoire of this HDM atopic individual (Fig. 1).

Polyclonal T cell responses to Der p 2 peptides

Polyclonal T cells from the same individual were stimulated with overlapping peptides covering the complete sequence of Der p 2, at 10 and 30 μg/ml on two separate occasions 8 weeks apart (Fig. 3A and B). Only the maximal proliferation induced by either of the peptide concentrations is presented. Although quantitatively different at these time points, the polyclonal T cell response was directed towards similar regions of the protein and the immunodominant peptides were located at amino acids 21 – 35 and 71 – 86 (Fig. 3A and B). When the overall magnitude of the proliferation was higher, T cell responses were also observed to the peptides spanning the flanking sequences 11 – 25 and 81 – 96. Nevertheless, relative to the background response of T cells cultured in medium alone, no other regions of the protein induced marked proliferation. The difference in the magnitude of proliferation observed on the two occasions may reflect frequency of environmental exposure to allergen.

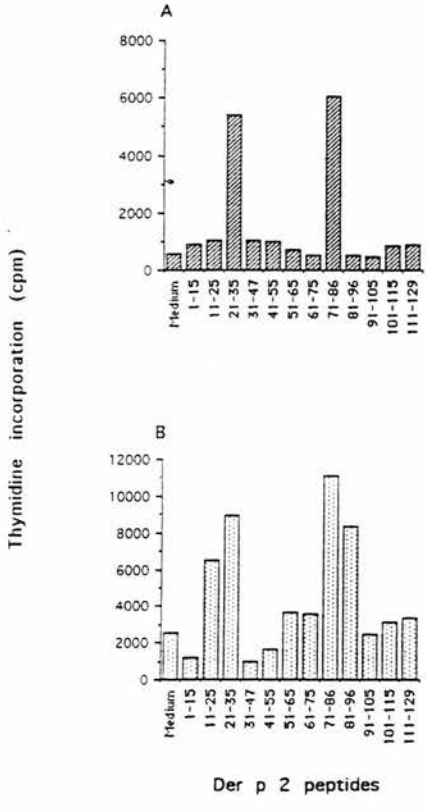

Fig. 3. Polyclonal T cell responses of a HDM atopic individual to overlapping peptides of Der p 2, measured 8 weeks apart.

Inhibition of the antigen-dependent responses of Der p 2 specific T cell clones

In order to define the subsets of HLA class II molecules restricting antigen recognition by the Der p 2 specific T cell clones, mAbs reactive with conserved sequences of HLA-D region molecules were added over a concentration range to T cell proliferation assays. Each individual T cell clone was cultured with its specific peptide (Fig. 2) at the optimal concentration to induce maximal proliferation. Antigen-dependent responses of the T cell clones AC1, AC2, and AC29 were inhibited in the presence of the anti-HLA-DR antibody (L243), whereas the anti-HLA-DQ (SPV-L3) and anti-HLA-DP (B7/21) antibodies had minimal effects (Fig. 4A – C). These results suggest that T cell clones AC1, AC2, and AC29 recognize HDM peptides in association with HLA-DR molecules. In contrast, the T cell clones AC3, AC5, and AC28 were blocked maximally by the anti-HLA-DQ antibody and no inhibition occurred in the presence of the anti-HLA-DP or anti-HLA-DR antibodies (Fig. 4D – F). The anti-DP antibody used in this study has been shown to block antigen presentation to other HLA-DP restricted clones (21). The results of these experiments imply that

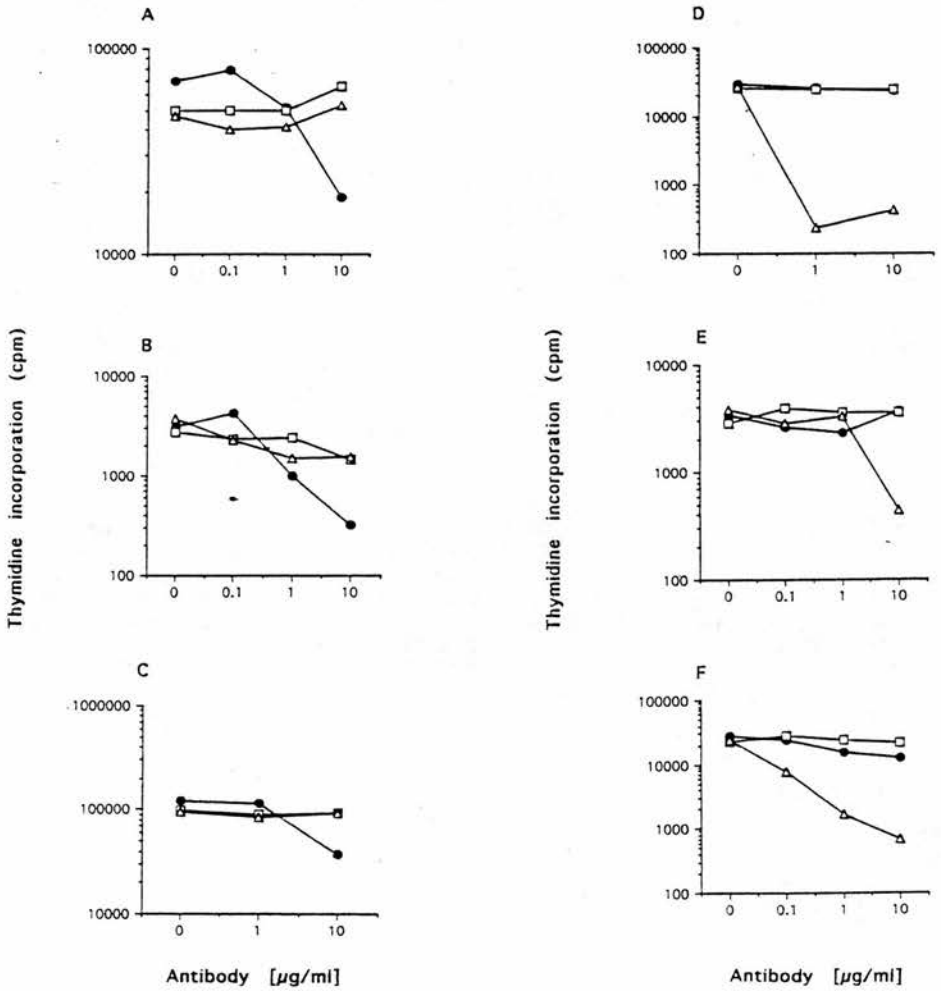

Fig. 4. Anti-HLA class II antibody inhibition of peptide induced proliferation by Der p 2 specific T cell clones. A, AC1; B, AC2; C, AC29; D, AC3; E, AC5; and F, AC28. Cloned T cells were incubated with peptide and APCs in the presence and absence of antibodies specific for HLA-DR (L243) (●), HLA-DP (B7/21) (□), and HLA-DQ (SPV-L3) (△).

Table 1. HLA-D region specificities of the panel of EBV-B cells used as APCs

Cell lines	HLA-DR specificities		HLA-DR alleles				HLA-DQ specificities	HLA-DQ alleles	
			DRB1	DRB3	DRB4	DRB5		DQA1	DQB1
YAR	DR4 Dw4	DR53	0402	—	0101	—	DQ8(3)	0301	0302
SSTO	DR4 Dw13	DR53	0403	—	0101	—	DQ8(3)	03	0302
HIN-ND	DR4 Dw15	DR53	0405	—	0101	—	DQ4	03	0401
DBB	DR7	DR53	07	—	0101	—	DQ9(3)	0201	03032
SWEIG	DR11(5) Dw5	DR52 Dw25	1101	0202	—	—	DQ7(3)	0501	0301
SPOH	DR11(5) Dw'DB2'	DR52 Dw25	1101	0202	—	—	DQ5(1)	0102	0502
JVM	DR11(5) Dw'JVM'	DR52 Dw25	1102	0202	—	—	DQ7(3)	0501	0301
RML	DR16(2) Dw22		1602	—	—	0202	DQ7(3)	0501	0301
COX	DR17(3) Dw3	DR52 Dw24	0301	0101	—	—	DQ2	0501	0201

HLA-DQ class II molecules may restrict antigen recognition by the Der p 2 reactive T cell clones AC3, AC5 and AC28.

HLA class II restriction specificity of Der p 2 reactive T cell clones

Based on the results of the serological inhibition studies described above, the HLA class II restriction specificity of the Der p 2 T cell clones was further investigated using APC panels consisting of HLA-typed, EBV-B cell lines selected for their expression of either HLA-DR or -DQ molecules (Table 1). In order to control for differences in the antigen concentration required to induce maximum proliferation of the individual T cell clones to their specific peptides, the dose ranges used in these HLA class II restriction experiments (Figs 5 and 6) were varied. The T cell clones AC1, AC2, and AC29 which were inhibited by the anti-HLA-DR antibody, showed similar patterns of proliferation to the APC panel (Fig. 5A – C). They responded in a dose-dependent manner to peptide presented by the autologous EBV-B cell lines [ACE: DR7, DR11(5), DQ2, DQ7(3)] or the EBV-B cell line homozygous for DR11 Dw5 (SWEIG; DRB1*1101). The other B cell lines tested, including JVM (DR11(5) Dw'JVM'; DRB1*1102) and DBB (DR7) failed to present antigen to the three T cell clones (Fig. 5). In the absence of both antigen and cloned T cells background proliferation by the B cell line JVM exceeded 4000 c.p.m. Nevertheless, this B cell line served as a valuable control for cross reactivity between the DR11 alleles.

Those T cell clones AC3, AC5, and AC28, inhibited by the anti-DQ antibody, were also investigated for their ability to respond to antigen using selected EBV-B cell lines. Dose dependent proliferation of the T cell clones AC3, AC5, and AC28 was induced to peptide presented by SWEIG, RML and ACE or FME, all of which have the DQB1*0301 allele (Fig. 6A – C). For the T cell clone AC28 the EBV-B cell line FME was used as positive control (Fig. 6C). This B cell line is histocompatible with SWEIG and ACE at the DR and DQ loci. Although ACE, FME, and SWEIG express the same DRB1 gene product (DRB1*1101), RML differs in that it expressed DRB1*1602. This supports the serological inhibition studies that these T cell clones may be restricted by HLA-DQ class II molecules. None of the T cell clones responded to antigen in the presence of the B cell line, COX, which shares the other DQB1 allele (DQB1*0201) expressed by ACE but differs in its expression of DR (DRB1*0301). However, the failure of COX to present peptide despite expressing the same DQα chain as SWEIG and RML (DQA1*0501) suggests that the α chain alone does not determine recognition. The B cell line SPOH which expresses DQA1*0102 and DQB1*0502 failed to present peptide to the T cell clones, although at a supraoptimal peptide concentration (10 μg/ml) limited proliferation of the T cell clone AC28 was induced (Fig. 6). SPOH and SWEIG share the same DRB1*1101 allele and are defined serologically as DR11(5). However, SPOH failed to present antigen to the T cell clones AC3, AC5, and AC28 (Fig. 6), but was able to induce antigen-dependent proliferation of the DRB1*1101 restricted T cell clone AC2 (data not shown). Collectively the results of the APC panel experiments and the ability to inhibit antigen-dependent proliferation with anti-HLA-DQ antibody suggest that DQ class II molecules function as restriction elements in T cell recognition of Der p 2.

*Molecular models for the β chains of HLA-DRB1*1101 and HLA-DQB1*0301*

The primary amino acid sequences of HLA-DRB1*1101 and HLA-DQB1*0301 are shown in Fig. 7. Molecular modeling of the β chains of these HLA-D molecules demonstrated that there are 15 amino acid differences between these two alleles located in the antigen combining site. In HLA-DQB1*0301 these residues are: Tyr9, Phe11, Ala13, Thr28, Ala38, Tyr47, Val67, Arg70, Thr71, Glu74, Leu75, Val78, Glu86, Leu87, and Thr90. In HLA-DRB1*1101 these residues are: Glu9, Ser11, Ser13, Asp28, Val38, Phe47, Phe67, Asp70, Arg71, Ala74, Val75, Tyr78, Gly86, Glu87, and Phe90. In addition to these potentially functional differences there are 25 further amino acid changes between these two alleles in the first 90 amino acids of the mature β chain (55.5% amino acid identity).

Discussion

The analysis of allergen specific IgE antibodies and T cell responses in the periphery of HDM atopic individuals has demonstrated that the group 2 allergens (Der p 2 and Der f 2) of HDM are a major target of immune recognition (4,8,7,14). The study reported here was designed to investigate in detail the heterogeneity of the Der p 2 reactive T cell repertoire in a HDM allergic patient. Analysing the antigen and restriction specificity of cloned T cells we have identified HLA-DR and -DQ restricted T cell epitopes, one of which is able to bind to both DR and DQ molecules.

Extending the population study in which we demonstrated that the major T cell epitopes of Der p 2 are located in the regions

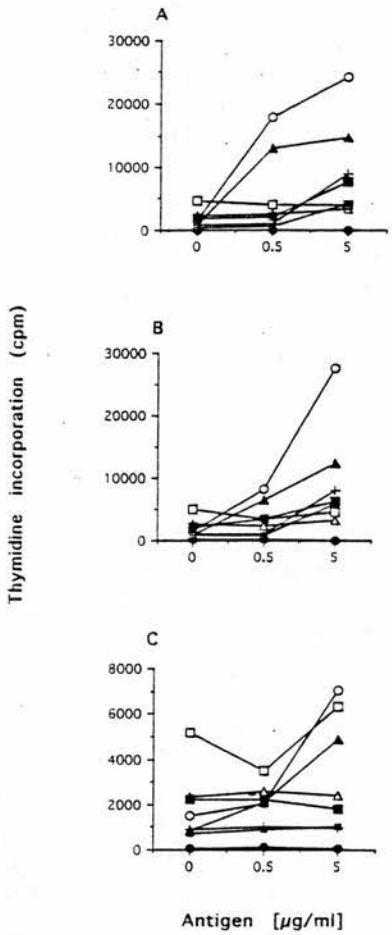

Fig. 5. HLA-DR class II restricted recognition of Der p 2 reactive T cell clones. The restriction of the T cell clones (A, AC1; B, AC2; and C, AC29) was determined using a panel of HLA-typed EBV B cells as APCs: DBB (△), SWEIG (▲), JVM (□), YAR (■), SSTO (+), and HIN-ND (⊞). Control responses of antigen-dependent proliferation in the presence of the autologous EBV-B cell line, ACE (○), and the T cell clone to antigen alone (●) were determined.

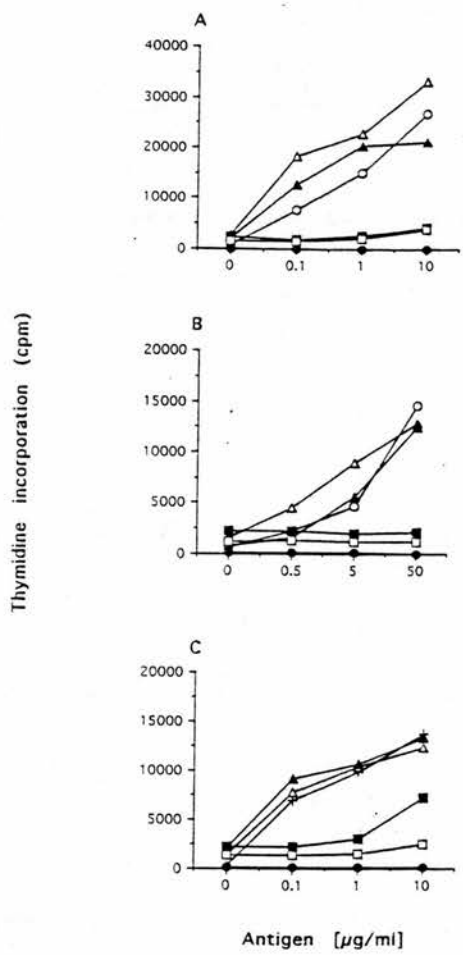

Fig. 6. HLA-DQ class II restricted recognition of Der p 2 specific T cell clones. The restriction of the T cell clones (A, AC3; B, AC5; and C, AC28) was determined using a panel of HLA-typed EBV-B cells as APCs: ACE (○), FME (+), RML (△), SWEIG (▲), SPOH (■), and COX (□). The background response of cloned T cells to peptide in the absence of APCs was also measured (●).

11 – 35 and 61 – 104 (14), a panel of T cell clones reactive with Der p 2 was isolated from the peripheral blood of a HDM atopic individual and their fine antigen specificity mapped using nested sets of peptides. The T cell clones (AC1, AC2, and AC3) all responded to residues 22 – 40, but there were minor differences in their pattern of proliferation to the peptides 25 – 37 and 28 – 40. This suggests that they recognize closely related epitopes in this region of the molecule. Three additional T cell epitopes were located within the regions covered by residues 16 – 31, 82 – 100, and 111 – 129. Analysis of the polyclonal T cells to overlapping peptides for the entire Der p 2 protein suggests that the epitopes identified by the T cell clones represent many of the antigen specificities of the overall peripheral T cell repertoire. However, comparison with the polyclonal response reveals the presence of additional epitopes. Therefore, it is important to include analysis of the specificity of polyclonal T cells in the investigation of the overall repertoire reactive with HDM. The practical application of developing peptide-based immunotherapy requires both mapping of the major T cell epitopes and identification of the HLA molecules regulating T cell recognition of HDM allergens. This

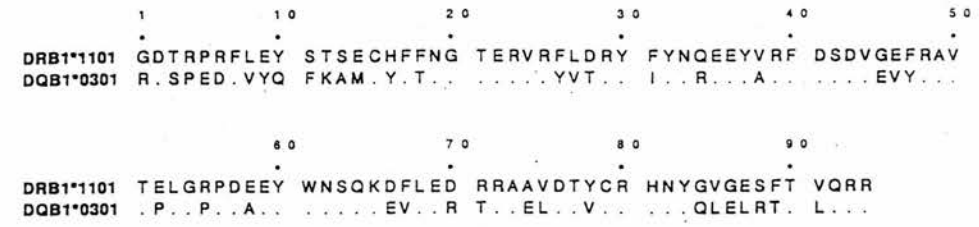

Fig. 7. Primary amino acid sequences of the first domains of DRB1*1101 and DQB1*0301.

prompted us to investigate the HLA class II restriction specificity of the Der p 2 reactive T cell clones in addition to antigen recognition by the polyclonal T cells. In serological inhibition studies we observed that antigen-dependent proliferation of the Der p 2 (22 – 40) specific T cell clones AC1 and AC2 was inhibited by the anti-HLA-DR framework antibody, whereas the T cell clone AC3, also specific for residues 22 – 40, was blocked by the anti-HLA-DQ antibody. Likewise, the T cell epitopes 16 – 31 and 111 – 129 recognized by T cell clones AC5 and AC28 respectively were inhibited by the HLA-DQ specific antibody. The remaining T cell epitope defined in this study, Der p 2-(82 – 100), recognized by clone AC29, appeared to be HLA-DR restricted. EBV-B cell lines selected for their expression of specific HLA-D region gene products were used as APCs and the class II restriction specificity of the Der p 2 reactive T cell clones was further analysed. Using this approach it was confirmed that T cell recognition of Der p 2-(16 – 31) and Der p 2-(111 – 129) is restricted by HLA-DQB1*0301 and Der p 2-(82 – 100) by HLA-DRB1*1101, whereas it appears that Der p 2-(22 – 40) is presented by both DR and DQ molecules of these specificities. The relevance of the DRB1*1101 class II molecules in the regulation of HDM allergic inflammation is not only limited to the recognition of Der p 2 as it has been reported that residues 94 – 104 of Der p 1 may be presented by this allele (20). The possibility of an identical epitope restricted by HLA-DRB1*1101 and HLA-DQB1*0301 is of considerable interest. However, due to the length of this region and the highly dissimilar chemical nature of the peptide binding clefts of these alleles it would appear unlikely that an identical epitope would bind to these two alleles. In the antigen combining site alone, there are amino-acid substitutions at 15 positions between HLA-DRB1*1101 and HLA-DQB1*0301 β chains. When the dissimilarity of the monomorphic HLA-DR α chain and the polymorphic HLA-DQ α chain are also considered (reviewed in 26), the differences between these two molecules make it unlikely that they are both able to bind an identical epitope. It is more likely that this region fortuitously contains two epitopes, one of which conforms to the HLA-DRB1*1101 motif, the other to the HLA-DQB1*0301 motif. This phenomenon of overlapping epitopes bound by different MHC molecules has recently been observed in the MHC class I restricted cytotoxic T lymphocyte response to the influenza virus nucleoprotein in an HLA-B*2702/B8 individual (27) in a highly promiscuous HLA-DR binding malarial peptide (28) and also in epitopes observed in a highly immunogenic region of the Der p 1 protein (21).

There are reports of associations between the expression of particular HLA-DQ class II molecules and responder status to specific allergens (29 – 31). From a genetic epidemiological study on cedar pollinosis in Japanese subjects it was revealed that the lack of specific IgE in the nonatopics is in linkage with HLA-DQw3 and that non-responsiveness is mediated by cedar pollen specific $CD8^+$ T cells (29). Similarly, a decrease in HLA-DR4 and HLA-DQw3 alleles has been observed in individuals who develop IgE antibodies to melittin or phospholipase A2 (30). Therefore, it appears that DQw3 may have a protective role in controlling IgE responses. However, an independent analysis, also in the Japanese population, reported that the frequency of HLA-DQw3 was increased in patients with rhinitis induced by cedar pollen and *D. farinae* (31), but as yet no HLA-DQ class II linkage with HDM allergic immune responses has been established for Caucasians (32,33).

The results reported here demonstrate that for a given HDM atopic individual a minimum of four T cell epitopes are recognized in Der p 2. Furthermore, these T cell epitopes appear to bind to either HLA-DR or -DQ molecules and, in the case of residues 22 – 40, binding to both subsets of HLA class II proteins was observed. Over a period of 8 weeks, we have examined polyclonal T cell responses to Der p 2 for this same individual chronically exposed to allergen and have failed to detect major differences in the pattern of peptide recognition. Plasticity in the T cell repertoire reactive with HDM allergens and the ability of major T cell sites to bind to both HLA-DR and -DQ molecules suggests that selected peptides may have a potential application in regulating the severity of the clinical symptoms of HDM allergic disease.

Acknowledgements

This work was supported by grants from the Wellcome Trust, the Medical Research Council, England, and Tripos Associates. R. O'H. is a recipient of a Wellcome Senior Research Fellowship in Clinical Sciences.

Abbreviations

APC	antigen presenting cells
EBV-B	Epstein – Barr virus transformed B cell
HDM	house dust mite
PBMC	peripheral blood mononuclear cells
[^{3}H]TdR	tritiated methyl thymidine

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1.4 MOLECULAR INTERACTIONS BETWEEN TCR, MHC CLASS II AND PEPTIDE OR SUPERANTIGEN MOLECULES

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Cell, Vol. 52, 515–523, February 26, 1988,

Structural Model of HLA-DR1 Restricted T Cell Antigen Recognition

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Summary

Two human helper T cell determinants in influenza have been identified, one in the hemagglutinin and the other in the matrix protein (M1). Both were shown to be DR1 restricted by using transfected L cells to present antigen. Comparison of the sequences of the two peptides revealed a similar pattern that could account for their DR1 specificity if the peptides adopt a helical conformation. The model was supported by the demonstration that hybrid peptides, composed of the amino acids that interact with DR1 from one determinant and the residues that interact with the T cell receptor from the other, were recognized by each clone. The generality of the motif was confirmed by the finding that DR1 individuals respond to a ragweed peptide containing the defined pattern.

Introduction

A unified model for T cell recognition of proteins has developed from recent experimental results from several laboratories. The observation that histocompatible cells are lysed when incubated with peptide fragments of influenza virus by both human and murine cytotoxic T cells (Townsend et al., 1986) demonstrated that cytotoxic T cells as well as helper T cells recognize processed antigens in conjunction with a MHC molecule. The many similar features in the response of CTLs to peptides with those of helper T cells taken together with the shared usage of identical genetic elements by the T cell receptors on the two populations of lymphocytes (Davis, 1985) argue that helper and cytotoxic T cells recognize protein antigens by a common mechanism.

Our understanding of the molecular basis of antigen recognition has been greatly enhanced by the finding that MHC class II proteins specifically bind peptides. Babbitt et al. (1985) demonstrated this by using a purified murine MHC class II molecule and a lysozyme peptide in equilibrium dialysis experiments; the class II–peptide complexes were subsequently shown to be sufficiently stable to be separated by using gel filtration techniques (Buus et al., 1986). This method has been used to extend the original observations to include several different MHC class II alleles (Buus et al., 1987) and, recently, human MHC class II proteins (Jardetzky, unpublished data).

Once formed, the complex between class II and peptide is believed to be the entity specifically recognized by the antigen receptor on the T cell. Competition studies, initially performed on the cellular level (Guillet et al., 1986) and subsequently with purified class II proteins (Guillet et al., 1987), have implied that the class II and class I molecules examined have a single antigen-combining site. In addition, the ability of the MHC proteins to bind fragments of proteins appears to correlate with antigen-specific responsiveness, for the generation of both helper (Guillet et al., 1987) and cytotoxic (McMichael et al., 1986) T cells.

The recent solution of the three-dimensional structure of HLA-A2 has revealed further details of MHC–peptide interactions (Bjorkman et al., 1987a, 1987b). Polymorphic residues of MHC class I molecules are concentrated in a proposed peptide binding site whose sides are composed of two α-helices, with the strands of the β-pleated sheet forming the base. MHC class II molecules can be modeled by sequence homology to adopt a similar structure. However, in the latter case, the site is composed of residues of the helices and strands of the amino-terminal regions of both the α and β subunits.

Consistent with the model of MHC proteins acting as receptors for peptide antigens, the areas in proteins that are recognized by T cells have a number of common structural features. Berzofsky and DeLisi have analyzed the known helper epitopes and have postulated that helper T cells preferentially recognize amphipathic helices (DeLisi and Berzofsky, 1985; Spouge et al., 1987). Independently, we have noted that the majority of both helper and cytotoxic T cell epitopes contain two adjacent hydrophobic residues preceded by a charged residue or a glycine that appears to be necessary for recognition (Rothbard, 1986). Although differing in emphasis the two observations are not necessarily mutually exclusive, and provide additional evidence for similarities in the details of the antigen binding sites of the MHC molecules. The merit of each analysis has been demonstrated by their ability to identify previously undefined T cell determinants successfully (Cease et al., 1987; Lamb et al., 1987; Rothbard and Taylor, 1988).

Several groups have commented on similar features in the sequences of the determinants when the latter are segregated by restriction element, which may be respon-

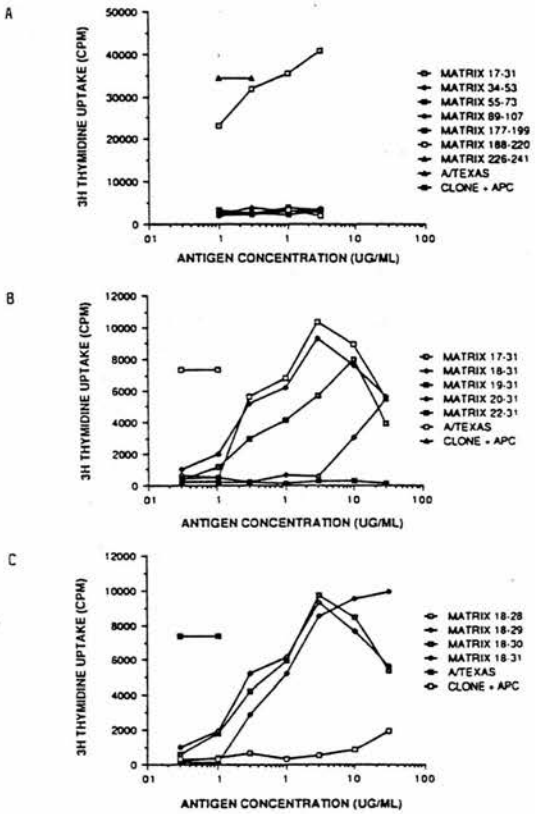

Figure 1. Definition of Matrix Protein Determinant Recognized by Clone TLC 72

(A) Localization of antigenic determinant to sequence 17–31 within matrix protein. (B) Definition of amino terminus of the minimal peptide capable of maximum stimulation. (C) Determination of the carboxyl terminus of the peptide capable of maximum stimulation. T cells (5 × 10^4/ml) of clone TLC 72 were cultured with irradiated histocompatible PBMC (1.25 × 10^5/ml), with antigen at the concentrations indicated. Proliferation as correlated with [^{3}H]TdR incorporation was determined after 72 hr. The control proliferative responses of the T cells to A/Texas influenza virus (5 HAU/ml) and to presenting cells alone were determined.

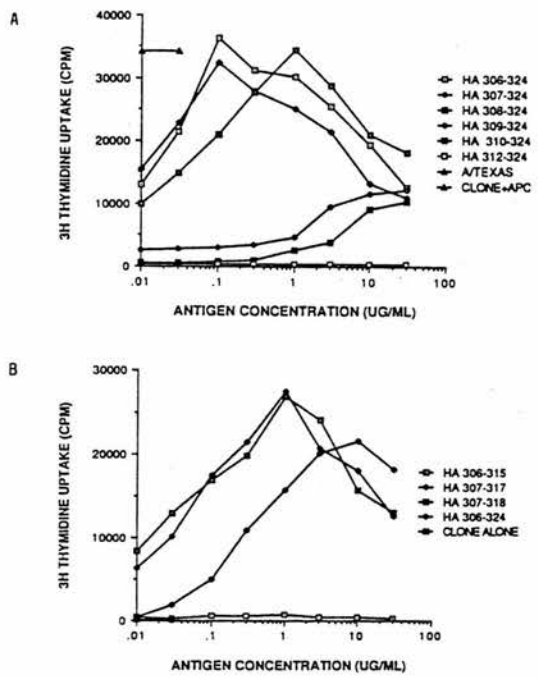

Figure 2. Definition of Hemagglutinin Determinant Recognized by Clone HA 1.7

(A) Definition of the amino terminus of the minimal hemagglutinin peptide capable of maximum stimulation. (B) Determination of carboxyl terminus of the minimal hemagglutinin peptide capable of maximum stimulation. T cells (5 × 10^4/ml) of clone HA 1.7 were cultured with irradiated histocompatible PBMC (1.25 × 10^5/ml), with antigen at the concentrations indicated. Proliferation was determined as described in the legend to Figure 1. The control proliferative responses of the T cells to A/Texas influenza virus (5 HAU/ml) and to presenting cells alone were determined.

sible for their specific binding to the MHC protein (Guillet et al., 1986; Buus et al., 1987; Rothbard and Taylor, 1988). However, as provocative as such allele-specific subpatterns are, they have yet to be demonstrated experimentally.

In this paper we have defined two DR1-restricted helper T cell epitopes in influenza, one in the hemagglutinin and the other in the matrix protein. The restriction was established by demonstrating that each clone was stimulated when the peptide antigens were presented by L cells transfected with the α and β chains of DR1. Comparison of the sequences of the two peptides revealed a similar pattern that could account for their specific binding by DR1. The model was successfully tested by demonstrating that hybrid peptides stimulated the appropriate T cell clone. The hybrid peptides were composed of the amino acids that interact with DR1 from one determinant and the residues that interact with the T cell receptor from the other. Finally, the generality of the pattern was examined by analyzing the ability of DR1 individuals to respond to a ragweed peptide containing the motif.

Results

Definition of Two DR1-Restricted T Cell Determinants

Seven peptides containing motifs characteristic of the majority of T cell epitopes (Rothbard and Taylor, 1988) within the sequence of the matrix protein (M1) of influenza were screened for their ability to stimulate a matrix-specific human helper T cell clone. The specificity of the clone, TLC 72, for the matrix protein has been previously described (Lamb et al., 1982b). Clone TLC 72 was stimulated only by the peptide corresponding to residues 17–31 (Figure 1A). The minimum sequence necessary for maximum stimulation was defined by testing nested sets of peptides that differed in length. Peptides with a common carboxyl terminus, but differing at their amino terminus, were used to stimulate the clone. Residues 18 and 19 were

Table 1. Proliferative Response of DR1-Restricted T Cell Clones to Antigen Presented by L Cells Expressing DR1

T Cell Clone	Antigen-Presenting Cells	Antigen	Response (cpm ± % SEM)
HA 1.7[a]	–	–	520 ± 31[b]
	DR1+ PBMC	–	304 ± 10
	DR1+ PBMC	HA 306–324	19,051 ± 2
	EBV-transformed DR1+ B cells	–	732 ± 23
	EBV-transformed DR1+ B cells	HA 306–324	11,771 ± 7
	DR1α DR1β–transfected L cells	–	253 ± 23
	DR1α DR1β–transfected L cells	HA 306–324	9,396 ± 2
	L cells	–	200 ± 15
	L cells	HA 306–324	285 ± 12
TLC 72[c]	–	–	112 ± 5
	DR1+ PBMC	–	176 ± 9
	DR1+ PBMC	MAT 18–30	6,022 ± 8
	EBV-transformed DR1+ cells	–	633 ± 3
	EBV-transformed DR1+ cells	MAT 18–30	8,668 ± 5
	DR1α DR1β-transfected L cells	–	607 ± 4
	DR1α DR1β-transfected L cells	MAT 18–30	15,751 ± 4
	L cells	–	281 ± 4
	L cells	MAT 18–30	144 ± 9

[a] Clone HA 1.7 (5×10^4 cells/ml) was cultured with and without HA peptide 306–324 (0.3 μg/ml) in the presence of irradiated histocompatible PBMC (1.25×10^5/ml), autologous EBV-transformed B cells (10^5/ml), or mitomycin C–treated murine L cells transfected with DR1α DR1β.
[b] Proliferation was measured as described in Figure 1.
[c] Similar culture conditions were used for clone TLC 72 except that matrix 18–30 (3 μg/ml) was the stimulating antigen.

shown to be required for maximal stimulation (Figure 1B). Serine-17 is not essential. Similar experiments examining the carboxy-terminal residues reveal that glutamic acid-29 is necessary but residues 30 and 31 are not, defining the minimum epitope to be 18–29 (Figure 1C).

Previous experiments with panels of antigen-presenting cells have suggested that clone TLC 72 was restricted by DR1 (Eckels et al., 1982). Another clone, HA 1.7, reactive with the hemagglutinin of influenza and isolated from the same individual, was shown to be restricted by the class II determinants DR1/DQw1 on panel studies (Eckels et al., 1984). The determinant for clone HA 1.7 had been partially characterized in earlier experiments to be present between residues 306 and 329 (Lamb et al., 1982a). This sequence contained two potential T cell recognition motifs (Rothbard, 1986), between residues 308 and 311 and 316 and 319. To test whether either or both of these areas are necessary for stimulation of the clone, we initially synthesized 306–324. This peptide contains all possible decamers containing the patterns. As seen in Figure 2A, 306–324 was fully stimulatory. The cysteine at 306 was not necessary for recognition because 307–324 was equally recognized. However, removal of proline-307 and, more effectively, lysine-308 results in the loss of stimulation (Figure 2A). The carboxyl terminus could be reduced to include alanine-318 without any loss of recognition (Figure 2B), allowing us to localize the determinant to residues 307–318, which contained the pattern from 308–311.

Presentation of Peptide Antigens to DR1-Restricted T Cells by L Cells Expressing Human Class II Antigens

The identification of restriction elements for human T cell clones is complicated because of the microheterogeneity of the HLA-D region products and the shortage of locus-specific monoclonal antibodies. To overcome this problem, we used murine fibroblasts transfected with the α and β chains of DR1 to prove unequivocably that both clones are stimulated by the appropriate antigen in the presence of DR1 (Table 1). The matrix-specific clone TLC 72 proliferated when the peptide was presented by autologous B cells transformed with Epstein–Barr virus, DR1-positive peripheral blood mononuclear cells, and DR1-transfected L cells (Table 1). Similar results were obtained with the hemagglutinin-specific clone by using 306–324 of the hemagglutinin (Table 1). The proliferative responses were in all cases inhibited by monoclonal antibodies directed at monomorphic DR determinants (R. Lechler, V. Bal, J. Rothbard, E. Long, R. Sekaly, R. Germain, and J. Lamb, submitted). From the results of these experiments, we conclude that both clones are DR1 restricted.

Analysis, Synthesis, and Testing of Hybrid Hemagglutinin–Matrix Peptides

Previous analysis of known T cell epitopes has revealed the presence of both general and allele-specific subpatterns that have been used for successful prediction of several previously undefined determinants (Rothbard and Taylor, 1988). The two DR1-restricted epitopes can be aligned in a similar manner based on the two adjacent hydrophobic residues (Figure 3A). Such an alignment reveals additional similarities at other positions. Most strikingly, lysine-21 is aligned with lysine-311. Also, a third pair of hydrophobic residues occurs at a fourth position, leucine-28 and alanine-318. These residues, whose relative positions are 1, 4, 5, and 8, would be juxtaposed if the peptides were to adopt a helical conformation, and would form a conserved face of the helix (Figure 3B).

We have previously postulated that the similar residues

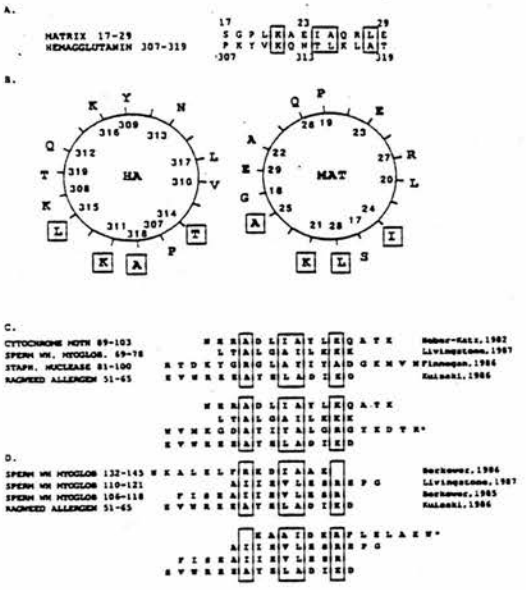

Figure 3. Sequence and Alignment of the Known DR1, E^d, and E^k T Cell Determinants

(A) Alignment of the two DR1-restricted peptides based on two adjacent hydrophobic amino acids. Boxed residues are those postulated to interact with DR1. (B) The two sequences displayed in a helical wheel. (C) Alignment of the defined E^k-restricted determinants, revealing their similarity with the DR1-restricted sequences. In the lower portion of the figure, the staphylococcal nuclease determinant has been reversed (•) to improve the alignment. (D) Alignment of the defined E^d-restricted determinants, revealing their similarity with the DR1-restricted sequences. As in (C), certain sequences have been reversed (•) to improve alignment.

in the two determinants constitute the principal residues interacting with DR1, while the amino acids composing the opposite face of the helix would interact with the T cell receptor. If true, this hypothesis would predict that those residues that interact with the restriction element could be exchanged between the epitopes without affecting recognition by either clone. Only those residues that are bound by the T cell receptor will be clonally specific. In Figure 3B, the area recognized by the restriction element would be the lower half of the helix, whereas the residues composing the upper facade would interact with the antigen receptor of the T cell. To test this hypothesis and to determine exactly which residues interact with DR1 and which are bound by the T cell receptor, two sets of peptides were synthesized and tested for their ability to be recognized by the T cell clones. In one set, the HAMAT series, amino acids from the hemagglutinin epitope were substituted into the matrix sequence. In the other set, MATHA peptides, amino acids from the matrix epitope replaced corresponding residues in the hemagglutinin determinant. The HAMAT series begins with three substitutions (HAMAT 1) and continues to HAMAT 9, which is identical to the original HA epitope except for one residue (Table 2A). The MATHA series is less extensive (Table 2B). MATHA 3 contains five substitutions, while MATHA 4 and 5 contain six and seven, respectively. The seven substitutions within MATHA 6 differ from those of MATHA 5 at two positions.

Table 2. Sequences of Hybrid Peptides Composed of Residues from the Two DR1-Restricted Epitopes

A	The HAMAT series											
	Matrix 17–29	S	G	P	L	K	A	E	I	A	Q	R	L	E
	HAMAT 1	S	G	Y	L	K	A	N	I	A	K	R	L	E
	HAMAT 2	S	G	Y	L	K	Q	N	I	A	K	R	L	E
	HAMAT 3	S	G	Y	L	K	Q	N	I	A	K	L	L	E
	HAMAT 4	S	K	Y	L	K	Q	N	I	A	K	L	L	E
	HAMAT 5	S	K	Y	L	K	Q	N	I	A	K	L	L	T
	HAMAT 6	P	K	Y	L	K	Q	N	I	A	K	L	L	T
	HAMAT 7	P	K	Y	V	K	Q	N	I	A	K	L	L	T
	HAMAT 8	P	K	Y	V	K	Q	N	T	A	K	L	L	T
	HAMAT 9	P	K	Y	V	K	Q	N	T	L	K	L	L	T
	HA 307–319	P	K	Y	V	K	Q	N	T	L	K	L	A	T
B	The MATHA series													
	Matrix 17–29	S	G	P	L	K	A	E	I	A	Q	R	L	E
	MATHA 3	P	K	P	V	K	A	E	T	L	Q	R	A	T
	MATHA 4	P	G	P	V	K	A	E	T	L	Q	R	A	T
	MATHA 5	P	G	P	V	K	A	E	T	L	Q	R	A	E
	MATHA 6	P	G	P	L	K	A	E	T	L	Q	R	A	T
	MATHA 4 A-17	A	G	P	V	K	A	E	T	L	Q	R	A	T
	MATHA 4 A-19	P	G	A	V	K	A	E	T	L	Q	R	A	T
	MATHA 4 V-19	P	G	V	V	K	A	E	T	L	Q	R	A	T
	HA 307–319	P	K	Y	V	K	Q	N	T	L	K	L	A	T

Residues enclosed in boxes correspond to the hemagglutinin sequence in the HAMAT series and the matrix sequence in the MATHA series.

The peptides were individually titrated with each clone and analyzed for their ability to stimulate. As seen in Figure 4A, HAMAT 1–3 were not recognized by HA 1.7, even at 100 μg/ml. In contrast, HAMAT 4 did stimulate the clone as well as the natural peptide at high concentrations, but was less efficient as the concentration of the peptide was decreased. Interestingly, HAMAT 5, 6, and 7 were not recognized even though they contained more of the HA sequence than HAMAT 4. HAMAT 8 and 9 were both recognized almost as well as the natural sequence.

To confirm that this result was not simply characteristic of HA 1.7, the reciprocal experiment was performed. However, TLC 72 did not proliferate in response to MATHA 3–6, including MATHA 4, which contains the reciprocal exchanges with HAMAT 4. One possible explanation for the failure of MATHA 4 to stimulate TLC 72 is that it contains the sequence of proline, glycine, proline, at the amino terminus. Proline is often stated to be incompatible with a helical conformation because the side chain is bonded to the backbone nitrogen, preventing its participation in hydrogen bonding and sterically preventing the correct conformational angles from being adopted (Richardson, 1981). This is not completely correct, because in the first three positions of a helix these hydrogen bonds are not formed. Therefore, although not favored, a proline can exist in the first turn of an α helix. However, in MATHA 4, the introduction of two prolines in the first three residues may have been sufficiently destabilizing to prevent an α-helical conformation from being adopted. To test this hypothesis, we synthesized three analogs of MATHA 4. One contained an

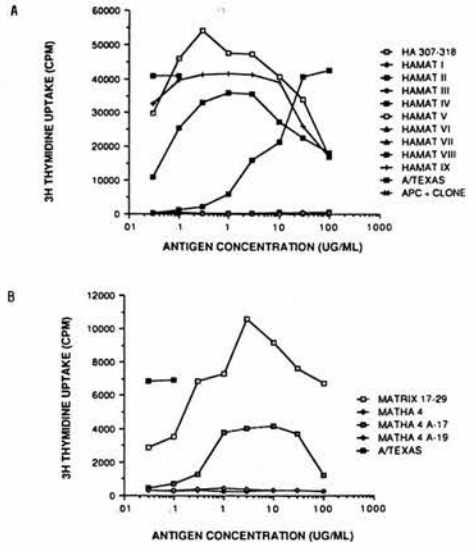

Figure 4. Response of Clones HA 1.7 (A) and TLC 72 (B) to Hemagglutinin and Matrix Hybrid Peptides

T cells (5 × 10^4/ml) were cultured with irradiated histocompatible PBMC (1.25 × 10^5/ml), with antigen at the concentrations indicated. Proliferation was determined as described in the legend to Figure 1.

alanine in place of proline-17, while the other two substituted an alanine and a valine for proline-19. If the peptide binds DR1 in the orientation we propose, then the substitution of an alanine at 17 will allow the peptide to adopt a helix and still retain the proline at 19, which we predict is recognized by the T cell receptor.

As seen in Figure 4B, only MATHA 4 with alanine-17 was stimulatory. As with HAMAT 4, it was not as efficient as the natural matrix sequence but it was, nonetheless, clearly recognized. In addition, the failure of the peptides containing alanine and valine at 19 is consistent with the proposed model. Finally, none of the HAMAT peptides were recognized by the matrix-specific T cell clone, nor did any of the MATHA peptides stimulate the hemagglutinin-specific clone (data not shown).

Identification of a DR1-Restricted T Cell Epitope in Ragweed Antigen E

To test the generality of the motif, we used a peptide from antigen E of ragweed, residues 54–61, to stimulate peripheral blood mononuclear cells (PBMC) from three DR1 individuals. We selected this peptide because not only did it contain the pattern, it also was immunogenic in H-2K and H-2D mice. As seen in Table 3, each of the individuals who recognized antigen E also responded to this peptide. PBMC from DR1 individuals whose T cells fail to proliferate in response to this protein also did not recognize the peptide, demonstrating that T cell proliferation was not due to a nonspecific mitogenic effect of the sequence.

Discussion

The recent demonstration that MHC class II molecules specifically bind peptide antigens has provided a molecular explanation for empirical analyses of the defined T cell epitopes, revealing that they share several features. Taken together, these ideas imply that there will be allele-specific patterns that correspond to the regions in each determinant that interact with the MHC proteins. To test these ideas, we have identified two DR1-restricted epitopes in influenza. The restriction element to which they bound was demonstrated by using L cells transfected with both chains of DR1 to present antigen. The details of the MHC–peptide interactions were examined by comparing the ability of mutated peptides to stimulate the clones relative to the natural sequences.

Assuming that the peptides share a common binding site, we examined the two DR1-restricted epitopes for common structural features. As seen in Figure 3, alignment of the two epitopes by the two adjacent hydrophobic residues revealed a similarity in the flanking residues. If the two lysines are considered to be residue 1, then there is similarity at relative positions 1, 4, 5, and 8. If the peptide adopted a helical conformation, these residues would be juxtaposed and would compose a common facade that could be characteristic of a DR1 binding site.

To determine whether the similarity in the epitopes had any structural validity and to analyze the conformation in which the peptide might bind MHC, we synthesized a family of peptides composed of residues from one determinant substituted into the other. The successful use of this strategy to produce a stimulatory peptide for each clone provides strong support for the proposal that both peptides bind DR1 in a helical conformation. The recognition of the hybrid peptides, which contained six modifications in each determinant, is especially remarkable considering the sensitivity of these and other peptide determinants to point mutations (data not shown; Sette et al., 1987; Allen et al., 1987).

The three-dimensional structure of the proposed antigen-combining site of histocompatibility antigens can ac-

Table 3. Proliferative Response of PBMC from HLA-DR1 Individuals to Ragweed Peptide 54–65

Donor	RaE 54–65	RaE	Medium	PHA
575 DR1,3	3,993 ± 21	1,489 ± 23	285 ± 5	150,037 ± 10
224 DR1,–	2,524 ± 10	8,545 ± 16	353 ± 26	82,864 ± 4
086 DR1,7	2,571 ± 5	6,869 ± 26	229 ± 29	79,808 ± 6

PBMC (5 × 10^4/well) were cultured with Ra peptide 54–65 (10 μg/ml), RaE (5 μg/ml), PHA, or medium. Proliferation as correlated with ^{3}HTdR incorporation was determined at 6 days.

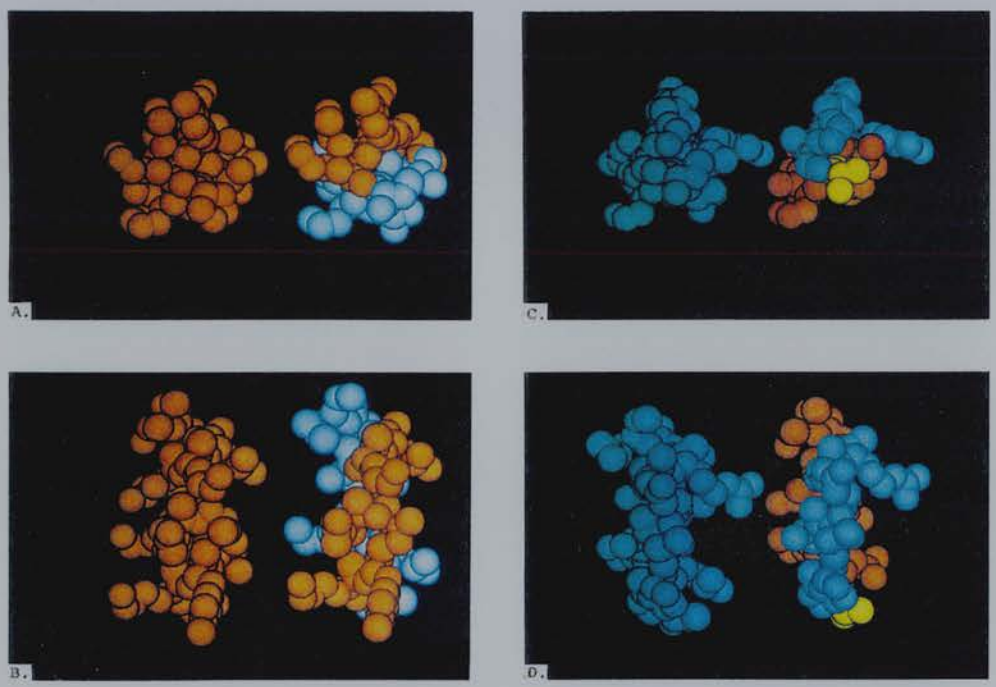

Figure 5. Space Filling Models of the Defined DR1-Restricted Determinants and the Stimulatory Hybrid Peptides in Helical Conformations

(A) Comparison of hemagglutinin 307–319 (left) with HAMAT 4 (right) viewed along the helical axes (z axis), with amino termini in the foreground. The orientation of the side chains is identical to that displayed in the helical wheel in Figure 3B. The residues proposed to interact with DR1 compose the lower facade of the helix, while those that interact with the T cell receptor are above (see text). (B) An orthogonal view of hemagglutinin 307–319 (left) and HAMAT 4 (right) to that of (A) (y axis), emphasizing the areas of the two peptides proposed to interact with the antigen receptor of HA 1.7 (see text). Relative to the orientation of the peptides in (A) and in Figure 4B, this view is from above. (C) Comparison of matrix 17–29 (left) with MATHA 4, with alanine (yellow) at position 17 (right), viewed along the helical axis (z axis) as in (A). (D) An orthogonal view of matrix 17–29 (left) and MATHA 4, with alanine (yellow) at position 17 (right), to that of (A) (y axis). As in (B), this view reveals that the residues necessary to interact with the antigen receptor of TLC 72 compose the face of the helix in the foreground. In each picture, residues corresponding to the hemagglutinin determinant 307–319 are red, while those of the matrix epitope, residues 17–29, are blue.

commodate a peptide in a helical conformation without any significant conformational changes in the protein (Bjorkman et al., 1987b). If we interpret our data in this context, the residues composing the described pattern would interact with the side chains of the amino acids of the HLA molecule pointing upwards from the β-pleated sheet, while the residues on the opposite face of the helix would point outward from the cleft and would interact with the T cell receptor. The amino acids of the epitope that are on the sides of the helix may, or may not, interact with the corresponding residues on the MHC helices depending on their length, polarity, and the complementary residues in the HLA protein. Consequently, even though the stimulatory hybrid peptide for each clone contained an identical number of substitutions on the upper facade of the helix, they do not necessarily make similar molecular contacts when bound by both receptors. For instance, the hemagglutinin-specific clone requires lysine-308 for stimulation, which had to be substituted by glycine in the stimulatory MATHA peptide. However, from its position on the helical wheel and space filling models (Figures 3B and 5), we believe that glycine-19 interacts with the restriction element and not the T cell receptor. Lysine is of sufficient length to extend out of the binding site and interact with the T cell receptor, while glycine's lack of a side chain would prevent any interaction.

The space filling models also provide a possible explanation as to why glutamic acid-29 and threonine-319 are not required for recognition even though from their position on helical wheels (Figure 3B) they appear to have a suitable orientation to interact with the T cell receptor. Both are the carboxy-terminal residues of the peptide and can be seen to be remote from the other clonal-specific residues. The diameter of an α helix is approximately 10 A. If we assume that the T cell receptor combining site is geometrically similar to that of an antibody, and the area of interaction is equivalent to that defined by the cocrystals of lysozyme and an antibody (30 × 20 A) (Amit et al.,

1986), then the receptor could contact the upper facades of all three helices, that of the epitope and the two of the MHC molecule. The length of the interaction along the helices would then be of the order of 20 A, consistent with the results with the hybrid peptides (Figure 5).

The space filling models also reveal that the shapes of the areas proposed to be recognized by the two receptors are distinctly different. As expected from the sequences of the two peptides, the faces of the helices interacting with the restriction element are quite similar (Figure 5). We expect, as with other instances of helices packing onto β-pleated sheets (Cohen et al., 1982), that the turns of the helical peptide will interdigitate with the four central strands of the MHC molecule. Consequently, we expect that variations in binding will occur, particularly if peptides can bind in either direction (vide infra). However, the two independently derived determinants described in this report appear to interact with the identical residues in DR1, suggesting that variations in binding might not be as large as one would initially expect.

That the hybrid peptides are not as effective in stimulating the clones is not surprising, primarily because residues from one helix cannot necessarily be substituted into another and still guarantee a helical conformation. This is vividly demonstrated by the failure of MATHA 4 to be recognized by the matrix-specific clone. Even though each peptide contained a proline in its first three amino acids, the hybrid synthesis resulted in two prolines in the same sequence which, as we discussed earlier, is unlikely to form a helix. In addition, in such an unprecedented situation where two macromolecular receptors bind the same ligand, subtle sequence-specific interactions may be important. These experiments also emphasize the potential diversity of molecular contacts with different T cell receptors. For although we predict a constant MHC–peptide interaction, the T cell receptor is still free to interact to varying degrees with residues exposed on the upper facade of the bound helical peptide. This would be consistent with the known differences and the fine specificity of T cell clones recognizing the identical peptide (Fink et al., 1986).

We have also provided preliminary evidence on the generality of the pattern of a positive residue—and three hydrophobic amino acids will be characteristic of DR1 responsiveness (Table 3)—by demonstrating that a ragweed peptide can stimulate peripheral blood lymphocytes from DR1 individuals. We were attracted to this peptide for several reasons. Ragweed was a useful system because a large number of individuals are known to respond to it. In addition to containing the pattern, this peptide was previously shown to be stimulatory in BALB/c and C3H.OH mice. A similar analysis of the defined I-E^k and I-E^d restricted epitopes (Rothbard and Taylor, 1988; Figures 3C and 3D) revealed a potential similarity to the two DR1-restricted determinants. These sequences could also be aligned to have a positively charged residue (either a lysine or an arginine) and two residues from a pair of hydrophobic amino acids, and be separated by six residues from a third. Conceivably, the similarity in the sequences of the determinants reflects common features in the antigen-combining site of the DR1, I-E^d, and I-E^k molecules. However, in order for the murine class II proteins to bind each of the known determinants in a manner similar to DR1, not only must each peptide adopt a helix, but in several cases the direction of the helix in the binding site is reversed. Such a situation would not necessarily violate the chiral nature of other known ligand–receptor interactions, because in a helical conformation the main chain amide nitrogens and the carbonyl oxygens are internally hydrogen bonded. Consequently, they would not interact with either the MHC molecule or the T cell receptor. In addition, the flexibility of the amino acid side chains on both the antigen and the two receptors could be sufficiently great to allow similar interactions to occur regardless of the relative orientation of the helix. Our results on the recognition of this peptide by DR1 individuals support these ideas and have prompted us to investigate further the similarities between DR1 and Ia E^d and E^k (Lechler et al., submitted).

We would not have identified many of these features if we had adopted the experimental strategy of point mutations used by the other two groups that have published analyses of peptide–MHC–T cell receptor interactions (Allen et al., 1987; Sette et al., 1987). As useful as these modifications have been to identify critical residues in MHC and T cell receptor binding, their interpretation is difficult. Ambiguity can arise because the two receptors bind a structure that results from the interactions between the amino acids of the antigen, and those interactions are not necessarily independent of each other. As we have shown, a single substitution at one position can remove all recognition, but a compensating change at another residue will restore binding. Our assumption that the peptide can be divided into two halves, one that is critical for binding to class II and one that binds to the T cell receptor, appears to be valid. Either half can be viewed as a recognition site that may be moved between determinants; however, mutations within each set often result in dramatic reduction in binding to either receptor.

The strategy of substituting alanine for the individual residues of the determinant has an additional shortcoming: only if the replaced amino acid dramatically differs in either size or polarity from alanine will a clear-cut distinction be seen (Allen et al., 1987; our unpublished data). For instance, with the hemagglutinin determinant, alanine can be successfully substituted at each position except for lysine-311 without significantly affecting recognition (data not shown). Consequently, certain residues appear not to interact with either receptor by this analysis. In contrast, our results indicate that each amino acid is involved in binding to DR1, the T cell receptor, or both.

We are currently attempting to identify the exact interactions the peptides make with DR1 by measuring binding constants of these and other analogs to purified DR1 and by crystallographic studies of both DR1 and DR1–peptide complexes. Detailed structural information from an HLA atomic model combined with similar sequence analyses of both helper and cytotoxic epitopes will lead to similar allele-specific subpatterns that can be used to develop logical strategies to modulate the immune system.

Experimental Procedures

Preparation of Lymphocytes

Peripheral blood mononuclear cells (PBMC) obtained from healthy adults were isolated by centrifugation on a discontinuous gradient of Ficoll-Hypaque (Pharmacia) and were cryopreserved. For use in experiments, lymphocytes were resuspended in complete medium: RPMI 1640 supplemented with A^+ serum, 2 mM L-glutamine, and 100 IU/ml of penicillin/streptomycin.

Isolation of Antigen-Reactive T Cell Clones

The isolation and characterization of human T lymphocyte clones HA 1.7 and TLC 72 have been described previously (Lamb et al., 1982a, 1982b). Briefly, PBMC were stimulated for 6 days with influenza hemagglutinin (H3) or intact virus (H3N2) for HA 1.7 and TLC 72, respectively. Lymphoblasts were enriched on a Percoll density gradient (Pharmacia, Uppsala) and cloned by limiting dilution (0.3 cells per well in Microtest II plates, Falcon) in the presence of autologous irradiated (3000 rads) PBMC, viral antigen, and interleukin 2. At day 7, growing clones were transferred to 96-well flat bottom microtiter plates and subsequently, to 24-well plates. At each transfer the clones received filler cells, antigen, and IL-2. The clones were expanded and maintained by the addition of fresh IL-2 every 3–4 days, and filler cells together with specific antigen every 7 days. Prior to use in proliferation assays, the T cell clones were rested for 6 to 8 days after the last addition of filler cells.

Proliferation Assays

Cloned T cells (5×10^4 per ml) were cultured with soluble antigen in the presence of irradiated histocompatible PBMC (1.25×10^5 per ml), autologous EBV-transformed B cells (10^5 per ml), or mitomycin C–treated transfected murine L cells (10^5 per ml) in a total volume of 200 μl of complete medium in 96-well round bottom plates. Where L cells were used as presenting cells, the assays were performed in 96-well flat bottom plates. After 72 hr of incubation, tritiated methyl thymidine (1 μCi, [^{3}H]TdR; Amersham International, Amersham, U. K.) was added to the cultures for 8–16 hr and then harvested onto glass fiber filters. Proliferation as correlated with [^{3}H]TdR incorporation was measured by liquid scintillation spectroscopy. The results are expressed as mean counts per minute (cpm) plus or minus percentage error of the mean for triplicate cultures.

Peptide Synthesis, Analysis, and Purification

Peptides were synthesized by using solid phase techniques (Barany and Merrifield, 1979) on an Applied Biosystems Peptide Synthesizer with commercially available Pam resins, t-Boc protected amino acids, and commercially available reagents (Applied Biosystems, Foster City, CA). The peptides were cleaved from the resin and the side chain protecting groups were simultaneously removed with anhydrous hydrofluoric acid with anisole as a free radical trap. The side chain protecting groups were extracted with ether. Subsequently, the peptides were dissolved in 15% acetic acid, filtered from the resin, and lyophilized. The crude peptides were analyzed by HPLC on a C-8 reverse phase column (Aquapore RP-300, Brownlee Labs) and by amino acid analysis. Peptides that were not of greater than 90% purity as judged by analytical HPLC analysis were purified by using a preparative RP-300 column and a water–trifluoroacetic acid–acetonitrile gradient. Principal peaks were collected, lyophilized, and analyzed by amino acid analysis.

Cloned Genes

The DR1 α and β genes were full-length cDNA gene clones, described by Tonnelle et al. (1985). They were inserted into the pRSV.5 and pRSV.3 cDNA expression vectors, respectively (Gorman et al., 1983). The pRSV.5 vector contains the pSV2*gpt* gene (Mulligan and Berg, 1981).

DNA-Mediated Gene Transfer, Selection, and Cell Sorting

The thymidine kinase (TK) negative murine L cell subline DAP-3 was transfected with pairs of α and β genes together with a third plasmid encoding either the xanthine-guanine phosphoribosyltransferase (*gpt*) or the neomycin resistance gene. The standard calcium phosphate precipitation technique was used (Margulies et al., 1983) and successfully transfected cells selected in medium containing either MXH (mycophenolic acid, xanthine, and hypoxanthine) or G418. Colonies of transfectants were pooled, stained with appropriate monoclonal antibodies, analyzed by microfluorimetry, and repeatedly sorted by preparative microfluorimetry to achieve high levels of cell-surface MHC class II expression.

Mitomycin C Treatment of Mouse Fibroblast (L) Cells

After trypsinization, cells were washed in serum-free RPMI 1640. Up to 10^7 cells were suspended per ml of serum-free medium. Mitomycin C (Sigma) was added to a final concentration of 50 μg/ml. Cells were incubated at 37°C for 45 min, washed extensively in A^+/RPMI, and used in the proliferation assays.

Acknowledgments

We thank Janet Thornton and Ted Jardetzky for helpful discussions. We are also grateful to Dr. M. Contreras of the National Blood Transfusion Center in Edgeware for providing blood donors.

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Received October 13, 1987; revised December 21, 1987.

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International Immunology, Vol. 1, No. 5 0953-8178/89 $3.00

Structural analysis of a peptide – HLA class II complex: identification of critical interactions for its formation and recognition by T cell receptor

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Key words: major histocompatibility complex, influenza haemagglutinin, biotinylated peptides, cell surface binding assay, T cell recognition

Abstract

An assay for the binding of peptides to major histocompatibility complex (MHC) class II proteins on the surface of cells has been used to determine the relative importance of the amino acids composing an influenza haemagglutinin T cell determinant in binding. The important contact residues were identified by the effect substitution of each residue with biotinylated lysine had on the ability of the peptide to bind. The spacing of the critical residues within the peptide sequence was consistent with the central core, of approximately eight amino acids, adopting a helical conformation. The terminal residues were less constrained and might not be part of a regular conformation. Increasing the helical propensity of the determinant, by simply acetylating and amidating the peptide, resulted in an analogue that was able to stimulate a specific T cell clone at significantly lower concentrations than the natural sequence. A potential location for the peptide in the binding site was postulated based on the presence of complementary amino acids in the class II molecule and supported by screening a large number of peptide analogues for their ability either to bind the restriction element or to stimulate T cell proliferation.

Introduction

The antigen receptor of T lymphocytes recognizes a complex formed between peptide fragments of protein immunogens and cell surface glycoproteins encoded by either the class I or class II major histocompatibility genes (reviewed in 1,2). Binding of peptides to detergent-solubilized major histocompatibility complex (MHC) class II molecules initially was demonstrated using equilibrium dialysis (3), and subsequently the complex was shown to be sufficiently stable to allow its isolation using gel filtration chromatography (4–6). Recently, binding of biotinylated analogues of T cell determinants to MHC class II proteins on the surface of Epstein–Barr (EBV)-transformed B cells has been shown using fluorescent streptavidin and flow cytometry (7).

Two groups have attempted to define the orientation of peptide determinants when part of the complex by correlating the inability of natural determinants containing point substitutions to stimulate the T cell clone with their capacity to bind the restriction element. Substitution of every position in a lysozyme peptide with alanine generated a family of analogues whose ability to bind purified I-A^k and/or stimulate a T cell clone provided sufficient information to allow a helical conformation of the bound peptide to be postulated (8). However, the results of a more extensive study using a similar strategy to dissect a helper determinant in ovalbumin failed to identify residues unequivocally interacting with either macromolecule (9). Consequently, a regular conformation could not be identified, primarily because the majority of peptides containing single substitutions were still able to bind the class II protein.

An alternative method was used to examine the orientation of two DR1-restricted determinants (10). Assuming the two peptides bound in a similar location in the combining site and adopted

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Transmitting editor: Lawrence Steinman

Received 25 May 1989, *accepted* 26 June 1989

identical conformations, residues from one sequence were substituted for the corresponding amino acid in the second to generate hybrid peptides that were able to stimulate T cell clones specific for each peptide. In addition to identifying residues critical for clonally specific recognition, this approach provided information on the structural requirements for binding and placed constraints on the potential conformations the peptides could adopt when part of the complex. However, this strategy had the inherent weakness that the putative MHC contact residues were identified on the basis of sequence homology between the two peptides, and consequently, the hybrid peptides that were stimulatory were quite similar to the natural determinant.

In this report the ability to detect binding of biotinylated peptides to MHC class II proteins on cell surfaces was used to develop a model for the relative orientation and conformation of an influenza haemagglutinin (HA) peptide in the antigen-combining site of HLA DR1. Only when an unnaturally large amino acid was incorporated into each position of the peptide could important residues for binding clearly be identified. The peptide was placed in the binding site based on the presence of potential complementary contact residues in the MHC molecule and the effects of a number of analogues on T cell recognition and binding. The ability of the MHC protein to bind a large variety of peptides is discussed in the context of the model.

Methods

Peptides

The peptide representing residues 307–319 of HA (PKYVK-QNTLKLAT in standard one-letter code), which previously was shown to be recognized by DR1-restricted T cells (10), was the parent sequence for the analogues used in this study. Long-chain biotin (11) was placed unambiguously at each position by substituting lysine at the desired position, replacing lysines 308, 311, and 316 with arginine, and acetylating the α-amino group. The peptides were synthesized using standard solid-phase methods as previously described (10) on an Applied Biosystems 430A synthesizer, and biotinylated with excess sulphosuccinimidyl-6-(biotinamido)hexanoate (Pierce). The biotinylated peptides were purified by reversed-phase HPLC and analysed by amino acid analysis and fast atom bombardment mass spectrometry. Biotinylation was further confirmed by positive reaction with dimethylaminocinnamaldehyde (12).

Binding assay

The assay has been described in detail elsewhere (7). Briefly, two EBV-transformed B cell lines homozygous for HLA-DR1 Dw1, MAJA, and METTE, and the class II deficient EBV-B cell line, RJ 2.2.5 (13), were incubated at 3×10^5 cells per well with each biotinylated peptide (50 μM) in 96-well plates (200 μl) at 37°C for 4 h, followed by addition of fluorescein isothiocyanate (FITC)–streptavidin (4.22 μg/ml; Calbiochem). Cell surface DR expression was quantified by staining with fluoresceinated L243 anti-DR monoclonal antibody (14; Becton-Dickinson, 30 min, 4°C). After each incubation, cells were washed with PBS containing 0.1% bovine serum albumin. Stained cells were analysed by flow cytometry using a FACScan analyser (Becton-Dickinson). Only viable cells, identified on the analyser for their ability to exclude propidium iodide, were included in the analysis.

In inhibition studies, competing peptides (6-fold molar excess; 300 μM) or unlabelled L243 (hybridoma supernatant, final dilution 2-fold) were included in the assay. To determine whether differential proteolysis was a factor, a mixture of protease inhibitors (TPCK, 10 μg/ml; PMSF, 50 μg; leupeptin, 1 μg/ml; aprotinin, 1 μg/ml; soybean trypsin inhibitor, 10 μg/ml) were co-incubated with cells and the biotinylated peptide.

Measurement of helicity

The helicity of the natural peptide (HA 307–319) or derivatives that were either acetylated or amidated, or both, was measured by the magnitude of the negative Cotton effect at 222 nm in a range of water–trifluoroethanol mixtures. On the scale shown, $10\ M^{-1}\ cm^{-1}$ was taken to represent 100% helix. All peptides underwent simple helix–coil transitions as judged by the presence of isodichroic points around 203 nm and the minima in the circular dichroism (CD) spectrum at 222 and 207 nm for the helical, and at 196 nm for the unordered conformation. CD spectra were recorded on a Jasco J-600 spectropolarimeter at ~0.15 mg/ml of peptide in a 1 mm quartz cuvette. Absorption spectra of peptide stocks were recorded on a Hewlett-Packard diode array spectrophotometer. CD spectra of biotinylated peptides were measured in 50% phosphate-buffered saline, pH 7.2/trifluoroethanol.

Isolation of antigen-reactive cloned T cells

The isolation and characterization of T cell clone HA 1.7 reactive with HA 307–319 has been described in detail elsewhere (15). Briefly peripheral blood mononuclear leukocytes (PBMC) were stimulated with HA and cloned by limiting dilution in the presence of autologous PBMC, antigen, and IL-2. Cloned T cells were expanded by the addition of IL-2 every 3–4 days and filler cells together with antigen every 7 days. Prior to use in proliferation assays, the T cells were rested for 6–8 days after the last addition of filler cells.

Proliferation assays

Cloned HA 1.7 T cells (5×10^4/ml) were stimulated with peptide antigens in the presence of histocompatible irradiated PBMC (1.25×10^5/ml). After 60 h of incubation, tritiated thymidine ([^{3}H]TdR; 1 μCi/well; Amersham International, Bucks, UK) was added and the cultures harvested onto glass fibre filters 8–16 h later. Proliferation as correlated with [^{3}H]TdR incorporation was measured by liquid scintillation spectroscopy. The results are expressed as mean cpm for triplicate cultures. Background response to accessory cells in the absence of antigen was <500 cpm and in all cases the SEM was <20%.

Results and discussion

Identification of amino acids critical for binding

Previous experiments exchanging amino acids between two DR1-restricted determinants suggested that both peptides adopted an α-helix when bound (10). In this model, the side chains of the amino acids composing the upper face of a helical peptide extend out of the antigen-combining site, and the residues which form the opposite façade and the sides of the helix make important contacts with complementary residues of the HLA molecule. Consequently, substitution of individual

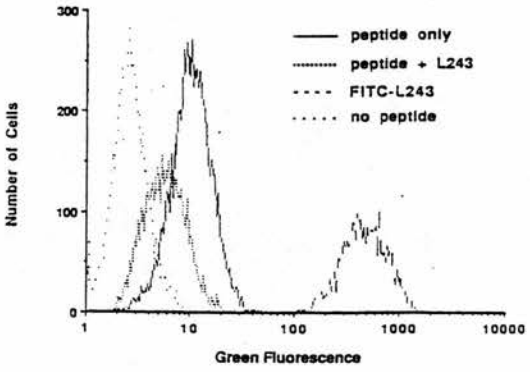

Fig. 1. Binding of a biotinylated analogue of HA 307–319 to EBV-transformed B cells. The DR1 Dw1 homozygous B cell line, MAJA, was incubated with an HA analogue containing LCB at the amino terminus and stained with FITC–streptavidin (——). Surface expression of class II DR proteins was quantified by incubation with a fluoresceinated anti HLA-DR monoclonal antibody, L243 (- - - -). Background fluorescence in the absence of biotinylated peptide was determined by incubation with streptavidin only (· · · · · ·). Co-incubation of the anti-DR monoclonal antibody and the biotinylated peptide resulted in a reduction of the fluorescent signal (· · · · · · · ·).

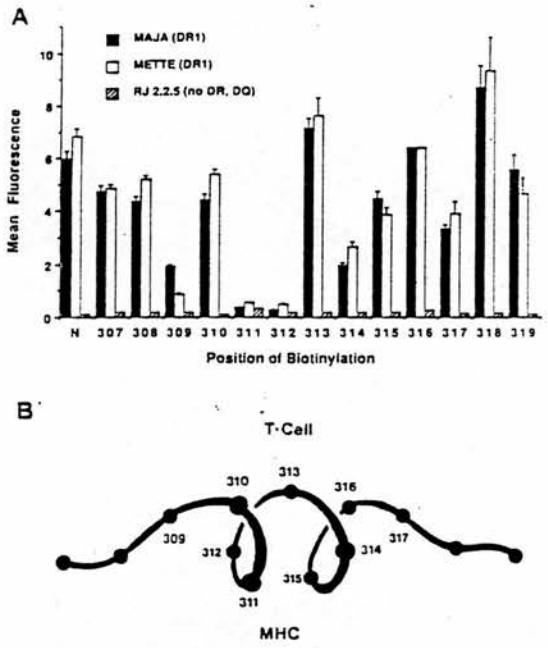

Fig. 2. Differential binding of analogues of HA 307–319, biotinylated at each position, to DR1-homozygous, EBV-transformed B cells. (A) Each peptide (50 μM) was incubated with transformed B cells homozygous for DR1 Dw1 [MAJA (■) and METTE (□)] or the DR-negative B cell line, RJ 2.2.5 (▨), stained, and analysed by flow cytometry. The relative amount of peptide bound to cells was judged by the intensity of green fluorescence per cell, averaged over all viable cells. The background as determined by the control incubations in the absence of biotinylated peptide was subtracted. The results shown are the average of three assays, with the SD displayed with error bars. (B) Model of the conformation adopted by the peptide when bound to DR1, deduced from the binding results shown in (A). Residues 309–317 are folded into an α-helix, with an orientation that permits residues 310, 313, and 316 to point away from, while 309, 311, 312, 314, 315, and 317 would be directed towards the antigen-combining site. The amino acids at both termini, which tolerate biotinylation, are not drawn as part of the helix because of their apparent conformational flexibility.

residues of the peptide by a bulky amino acid should differentially affect the stability of the complex depending on the steric requirements of each contact and the relative importance of each amino acid in binding. However, the structure of the antigen-combining site of the MCH molecule has evolved to bind multiple, unrelated peptides and therefore is expected to tolerate many point substitutions in a peptide with only slight effects on the apparent affinity of binding. This might be why previous studies (9) examining binding of ovalbumin peptide analogues to murine class II molecules have failed to define critical MHC contact residues unambiguously. However, an unnaturally large side chain might cause greater steric interference with binding than substitution with any natural amino acid, thereby allowing us to observe a greater reduction in binding upon altering a critical residue.

To test this hypothesis, a set of peptides containing a derivative of biotinylated lysine with an additional hydrocarbon spacer, long-chain biotin (LCB; 11), substituted for each residue of the HA peptide was synthesized. Binding of the analogues to the surfaces of DR homozygous EBV-transformed B cell lines was assayed by incubating peptide-treated cells with fluorescently labelled streptavidin and analysed by flow cytometry (7). When the HA peptide, biotinylated at the amino terminus, was incubated with the DR1-expressing cell line and exposed to FITC–streptavidin, a fluorescent signal ~5 times that present when the cells were incubated in the absence of the peptide was apparent (Fig. 1). Previous experiments have shown that the fluorescent signal arises from the direct interaction between the peptide and HLA-DR molecules on the surface of the cell and does not require internalization (7).

In this paper, biotinylated analogues have been used as probes to examine the relative importance of each residue in binding. When peptides containing lysine-LCB at each position were incubated with the cells, marked differences were seen in the resultant fluorescent signal (Fig. 2A). Strong fluorescence was present when lysine-LCB was placed either at the N terminus or substituted for proline-307, lysine-308, valine-310, asparginine-313, lysine-316, or alanine-318. In contrast, no detectable fluorescence was observed when peptides containing lysine-LCB at residue 311 and 312 were used, while substitution at 309, 314, 315, or 317 resulted in reduced fluorescence.

A loss of the fluorescent signal upon biotinylating any residue might occur because the ability of the peptide to bind to DR1 is reduced by the modification, or because the peptide still binds but with the biotinyl group sterically unavailable to streptavidin. In the latter case, no fluorescent signal should be apparent at any concentration, because steric inaccessibility should not depend on the amount of peptide in the assay. The appearance

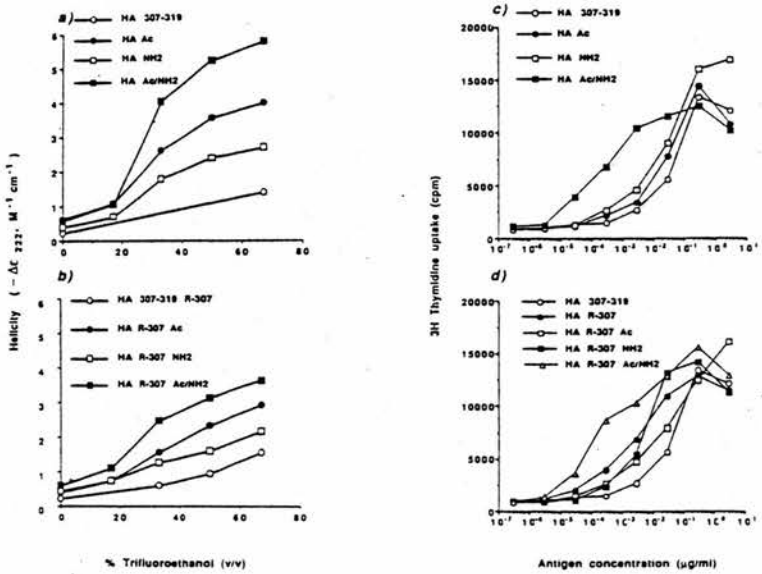

Fig. 3. Chemical modification of HA 307–319 resulted in both greater helical propensity and increased antigenicity. (a) Helicity of the natural peptide (HA 307–319), or derivatives that were either acetylated (HA Ac), amidated (HA NH2), or both (HA Ac/NH2), measured in varying water–trifluoroethanol mixtures. (b) Helicity of analogous HA 307–319 derivatives containing an arginine (R) residue at position 307. (c) T cell proliferation in response to acetylated and amidated analogues of HA 307–319. The response to the natural sequence is shown for comparison. (d) T cell proliferation to R-307 substituted analogues.

of a fluorescent signal when analogues containing lysine-LCB at 309, 311, and 312 were used at concentrations well above 50 μM (data not shown) indicated that biotinylation affected the apparent affinity of the analogues for DR1.

The ability of the analogues to bind to the restriction element on cell surfaces could have been affected by the biotinylation in a number of different ways: (i) by interfering with a critical contact between the peptide and the class II proteins; (ii) by altering the propensity of the peptide to adopt the conformation in which it bound to DR1; or (iii) by changing the susceptibility of the analogue to proteolytic degradation. The second possibility was unlikely because each of the 14 peptide analogues had indistinguishable CD spectra (data not shown) in trifluoroethanol (TFE)–water mixtures containing TFE concentrations at which the helical propensities of closely related, unbiotinylated HA analogues were significantly different (*vide infra*). The third possible explanation for the different fluorescent signals generated by each analogue, differential proteolysis, was more difficult to disprove. However, none of the low fluorescent signals was increased in the presence of a cocktail of protease inhibitors (TPCK, PMSF, leupeptin, aprotinin, and soybean trypsin inhibitor). Therefore the differences in fluorescence appear to arise from the varying capacity of the class II molecule to bind the analogues, reflecting the differential effect of biotinylation at each position on the affinity of the interaction.

If this interpretation is true, then the assay provides a quantitative measure of the involvement of each amino acid of the peptide in the formation of the complex: the lower the signal, the more important is the amino acid. Therefore we can conclude that tyrosine-309, lysine-311, glutamine-312, and, to a lesser extent, threonine-314, leucine-315, and leucine-317 contribute to the formation of the complex.

The peptide might bind to DR1 in a variety of ways. However, the distinct variations in fluorescence observed when the different analogues were assayed implied that the number of conformations and orientations of the bound peptide was limited. In all possible modes of binding, residues 311 and 312 form critical contacts with the restriction element, because biotinylation at these positions eliminated the fluorescent signal at the peptide concentration used. A further constraint on the possible conformations of the bound peptide was that the fluorescent profile peaked at every third residue (310, 313, and 316) within the central portion of the peptide, with significantly less fluorescence in between. The periodicity suggested that the central core adopted a helical conformation. However, the results were not consistent with the peptide being helical over its entire length because analogues containing lysine-LCB at 308 and 318 resulted in a strong signal. If the peptide adopted a perfect helix with 310, 313, and 316 pointing up, these residues should point down. The ability to tolerate substitution with biotinylated lysine at both ends of the peptide might be explained by an increased accessibility of the termini of the helix or by deviations from an ideal α-helix. A model based on this interpretation of the pattern of fluorescence is shown in Fig. 2B, consisting of a helical core

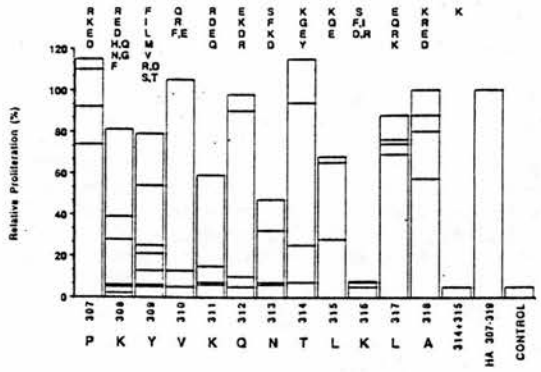

Fig. 4. The ability of peptide analogues of HA 307 – 319 containing substitutions to stimulate the HA-specific T cell clone. The proliferation assays were performed as described in the Methods. Proliferation to the substituted peptides is expressed as a percentage of the response observed to the natural sequence (HA 307 – 319) at the optimal concentration (0.3 μg/ml), although each peptide was tested over a dose range of 0.3 – 30 μg/ml. The horizontal axis represents the sequence of the natural determinant; monosubstitutions at each position are shown above the histogram in order of decreasing proliferation. Where the two peptides gave indistinguishable proliferation, they are shown on the same line. A disubstituted peptide containing two lysines at positions 314 and 315 is also included. The proliferative response of cloned T cells cultured with accessory cells in the absence of antigen is shown as a control.

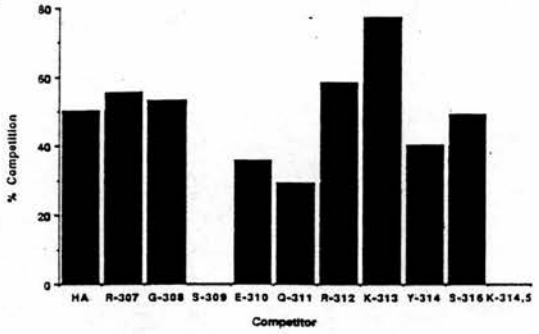

Fig. 5. Percentage competition of monosubstituted HA peptides for binding of the N-terminally biotinylated HA analogue to the DR1 Dw1 homozygous B cell line, MAJA. Cells were simultaneously incubated with 50 μM biotinylated peptide and 300 μM (6-fold molar excess) competitor peptide, stained, and analysed as described in the legend to Fig. 1.

(residues 309 – 317), with the two amino acids at each end of the peptide exhibiting greater conformational freedom and not modelled as part of the repeating structure.

Stabilization of a helical conformation results in increased antigenicity

If the HA peptide binds the restriction element as an intact or partial α-helix, then stabilization of this conformation should improve the potency of the peptide. Recent experiments have demonstrated that stabilization of the macrodipole of an α-helix can result in a significant increase in the helical content of relatively short peptides (16). One way to stabilize the macrodipole is to remove the charges at the amino and carboxyl termini of the peptide by acetylation and amidation.

The natural HA peptide and several analogues were examined for secondary structure content by CD in a range of water – TFE mixtures. The unacetylated and unamidated HA peptide exhibited only low helicity even at high TFE concentration (Fig. 3a), as judged by absorption of circularly dipolarized light at 222 nm. Amidation, acetylation and, to a greater extent, combined acetylation and amidation substantially increased the helicity of the HA peptide. Consistent with the hypothesis that the improved helical content was due to stabilization of the macrodipole, amidation and acetylation of an analogue with arginine at 307 also increased helical propensity, but less so than when the positively charged arginine was not the first residue in the peptide (Fig. 3b).

When tested in proliferation assays with the T cell clone, HA 1.7, the acetylated and amidated peptide stimulated the clone at approximately two orders of magnitude lower concentrations than the natural sequence (Fig. 3c). Replacement of the amino terminal proline by either lysine or arginine also resulted in more potent peptides (Fig. 3d), but the acetylated, amidated peptide containing arginine at 307 was not recognized better than the acetylated and amidated natural sequence. That the effects of exchanging proline for a positive charged residue and blocking the charged end groups were not additive indicated that the two factors are acting by separate mechanisms. The most likely explanation is that a positive charge at 307 results in a superior interaction with a residue in the binding site of DR1, but does not stabilize the macrodipole.

Recognition and competition by analogues containing point substitutions

To begin to understand the physical and chemical requirements at each position of the peptide for binding to DR1 and recognition by the T cell receptor of HA 1.7, sets of peptides containing point substitutions at each position in the sequence were synthesized. Each peptide was assayed over a concentration range of 0.03 – 30 μg/ml for its ability to stimulate the HA-specific clone. The proliferative responses relative to the natural sequence at 0.3 μg/ml are shown in Fig. 4. As in previous studies on both class I- and class II-restricted T cell recognition (9,17), relatively few substitutions resulted in peptides that were recognized better than the natural sequence (R, K-307; Q-310; K-314).

Several peptides containing a point substitution, which resulted in complete loss of recognition by HA 1.7, were tested for their ability to displace the natural sequence containing an amino-terminal LCB from the antigen-combining site of DR1 on the surface of an EBV-transformed B cell line (Fig. 5). Interestingly, with the exception of a peptide containing serine at 309, they all successfully competed to varying extents. Some of the best competitors were analogues that contained substitutions at positions shown to tolerate LCB and postulated to point outwards from the site (307, 308, 310, 313, and 316). Surprisingly, analogues containing substitutions at residues believed important for binding (311, 312, and 314) also bound sufficiently well to compete effectively.

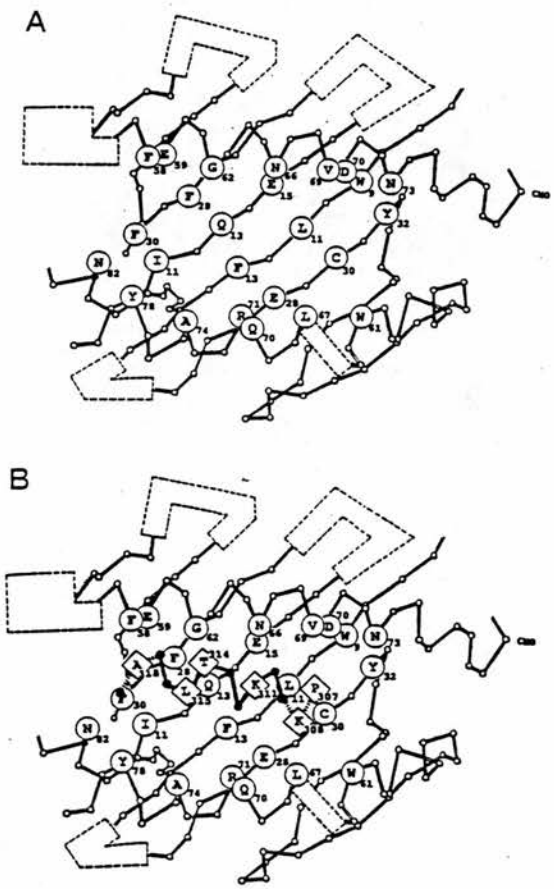

Fig. 6. Proposed model of the antigen-combining site of DR1 and the complex formed between the HA peptide and the class II protein. (A) The proposed antigen-combining site of DR1 modelled by homology to the published structure of HLA-A2 (18,19). The model is consistent with the alignment of class I and class II amino acid sequences by Brown *et al.* (20). The areas enclosed in dashed lines correspond to positions with either additions or deletions of residues between consensus class I or II sequences. (B) The proposed location of HA 307–319 in the combining site. The peptide is modelled as two turns of a perfect α-helix and placed in the site parallel with the β-chain helix. The flexibility at the termini suggested by the binding experiments is indicated by the broken lines connecting the terminal α-carbons of the peptide. The residues of the peptide believed to be facing down in the site are enclosed in diamonds.

Taken together with the earlier experiments on the ovalbumin peptide (8) and related studies on class I-restricted determinants (17), these findings indicate that there is no absolute requirement for a single amino acid in most positions in T cell determinants. Monosubstitutions in the peptide may change the detailed interactions with the MHC protein or the surface of the peptide–MHC complex as seen by the T cell receptor, but without significantly reducing the amount of peptide bound to the restriction element. Consequently, experiments using analogues containing point substitutions to determine relative orientation of the peptide can lead to results that are difficult to interpret. Only if, as shown above using long-chain biotinylated lysine, the substitutions are sufficiently drastic, will distinct variations in binding be apparent. This high degree of tolerance to sequence variation in the peptide might be expected for a binding site containing several amino acids of different physical character in close proximity to each residue of the agretope. In addition, the flexibility of the side chains in the binding site might allow the MHC protein to accommodate the steric effects of mono-substitutions.

Modelling a DR1–peptide complex

To identify a potential location of the peptide in the binding site, a model of DR1 (Fig. 6A) was generated based on the published HLA-A2 structure (18,19). As previously detailed (20), alignment of the MHC class I and II alleles, whose sequence has been determined, revealed that the two classes of proteins share a number of structural features.

The results of the experiments using biotinylated analogues indicated that the central core of the HA peptide bound in a preferential orientation and conformation. The dimensions of the proposed antigen-combining site are sufficient to allow a 13 amino acid peptide, folded as at least two turns of an α-helix, to be placed in the site with its helical axis approximately parallel with the helix of the α-chain of class II, as has been postulated previously for a lysozyme peptide (20). In such a model, the residues composing the lower façade of the helical peptide would be expected to pack closely onto the four central strands of the β-pleated sheet of the recognition element (Fig. 6B) as is characteristic of the helix – strand interactions in globular proteins (21–23). In this conformation, the amino acids composing the sides of the helical peptide can interact with either the residues of the helices of the MHC protein, those of the T cell receptor, or both.

If the orientation of the peptide relative to the MHC protein and the antigen receptor of the T cell was valid, as indicated by the exchange experiments (10) and the binding of the biotinylated analogues, then the proposed antigen-binding site should have complementary features that might allow the identification of contact residues in the ligand and its receptor.

The first position examined in the peptide was lysine-311. Empirical analyses initially identified this residue as potentially interesting (24) and the exchange experiments between determinants (10) and the binding assays have confirmed its importance. A positive amino acid at this position was advantageous for binding because a peptide containing arginine was recognized by the T cell clone, whereas substitution of lysine by glutamic or aspartic acid or glutamine resulted in either complete or significant loss of stimulatory activity. Biotinylation of this lysine greatly affected the ability of the peptide to bind DR1 and indicated that this residue most likely is buried in the complex. Buried positive charges are often involved in salt bridges in globular proteins (25), and together with the sensitivity of this residue to substitution, suggested that the lysine participates in an ionic interaction.

Two other positions in the peptide also exhibited a preference for a positively charged amino acid, although not as clearly as

at 311. Of the nine amino acids substituted for lysine-308, arginine was the best recognized. Substitution of the amino-terminal proline at 307 with lysine or arginine resulted in more potent peptide analogues, stimulating the clone at ~10 times lower concentration than the natural sequence (Fig. 4). The increased potency was not simply due to the removal of the proline at this position, because the other two substitutions (D and E) did not increase the activity.

The molecular surface of the combining site was examined for a pattern of buried negative charges that could act as counter-ions for positive charges at 311, 308, and 307 in the peptide. Because of its demonstrated importance in binding, lysine-311 was assigned a central position in the site near glutamic acid-15 of the α-chain. If the amino terminus of the peptide was orientated towards the strands of the β-chain, as shown in Fig. 6B, then lysine-308 was located so that it could interact with glutamic acid-28 of the β-chain. Alternatively, lysine-308 might interact with glutamic acid-59 of the α-chain if the direction of the peptide was reversed; however, only the orientation shown in Fig. 6B can account for the preference for a positively charged residue at position 307 because, in this configuration, residue 307 is near aspartic acid-70 of the α-chain.

The other residues shown to contribute to binding (314, 315, 309, and 312) were examined in the context of this tentative model. As shown in Fig. 6B, residues 314 and 315 of the peptide are located near the first and second strands of the α-chain. Interestingly, lysine could be present at either position with no deleterious effect on recognition. One possible rationalization for the tolerance of both hydrophobic and hydrophilic amino acids at these two positions is the diversity of interactions possible with the receptor. For example, if a hydrophobic residue is present at position 315 in the peptide, it can be tolerated because it could form good contacts with several hydrophobic residues in the class II binding site, namely phenylalanine-30 or -28, or isoleucine-11 of the α-chain, whereas if a polar residue is present it may bind because it interacts with a distinctly different, polar amino acid, glutamine-α13. However, there are limits to the ability of the protein to tolerate substitutions in the determinant. A peptide analogue containing lysine at both 314 and 315 did not compete with the biotinylated native sequence for binding (Fig. 5) and was not recognized by the T cell clone (Fig. 4), suggesting that some hydrophobic character is required at these two positions.

In addition to 311, amino acids at 309 and 312 appear to be critical for binding. When bound as shown in Fig. 6B, they are both directed towards the β-chain helix, with tyrosine-309 of the peptide close to leucine-67 and glutamine-312 juxtaposed to residue 71 in the model. Tyrosine-309 can be replaced by phenylalanine and be recognized by the T cell clone, but substitution with eight other amino acids resulted in significant loss of stimulation (Fig. 4). Substitution with serine at 309 also was the only point mutation that failed to compete with the biotinylated natural sequence, demonstrating that tyrosine at this position makes an important interaction with the restriction element (Fig. 5). When glutamine-312 was substituted with arginine all recognition was eliminated, whereas substitution with either aspartic or glutamic acid retained the ability of the peptide to stimulate the clone. The ability to tolerate a negative, but not a positive, charge was consistent with position 312 in the peptide interacting with arginine-71 of the β-chain. However, the limitations to the monosubstitution approach are highlighted by the observation that a substitution with lysine at position 312 did not eliminate recognition. While not necessarily disproving the proposed model, this fact serves to emphasize the degree of tolerance typical of the formation of the complex between the HA peptide and DR1.

Due to the small number of substitutions and the variation with which they differ from the native sequence at each position, only limited conclusions can be made about the tolerance for amino acid substitutions along the sequence. In addition, the ability of analogues of the same peptide containing point substitutions to compete with the natural sequence for binding demonstrated that there was significant flexibility in the formation of the peptide – MHC complex. Nevertheless, a tentative position for the peptide was identified based on potential interactions with residues in the site. The model has been constructed in which the peptide adopts a conformation with its central core of approximately eight amino acids as a fixed α-helix and the diversity of binding arises from flexible side chain interactions. Such a central core of residues in a T cell determinant is consistent with investigations examining the minimal requirements for T cell recognition and binding to MHC class I and II molecules (9,24). Outside of the central core of the antigen, the conformation of the peptide appears to be less constrained, which might contribute to differences in fine specificity of T cell clones recognizing the identical peptide sequence. The three most critical residues for binding are centrally located, one pointing towards the pleated sheet and two directed at the β-chain helix. Lysine-311 on the peptide was postulated to interact with glutamic acid-15 of the α-chain while glutamine-312 and tyrosine-309 are believed to contact arginine-71 and leucine-67 of the β-chain helix. The proposed contact residues in the MHC protein dramatically differ in their polymorphism. The glutamic acid is present in all DR proteins, whereas 67 and 71 of the β-chain are highly variable between alleles. Interestingly, positions 67 and 71 are two of the three mutations found in the A_β^{bm12} murine class II protein, which are known to change T cell antigen recognition dramatically (26,27). Also, position 71 has been identified as being associated with susceptibility or resistance to rheumatoid arthritis and pemphigus vulgaris (28). One possible explanation for their importance is that they are located in a position on the side of MHC helix where they contact not only the side chains on the antigen, but also potentially the antigen receptor of the T cell.

Although the biochemical and functional data reported here have permitted the generation of a working model, the true structure of peptide – MHC class II complexes will remain elusive until the crystal structure of a class II protein and of a pure class II – peptide complex have been solved. The importance of a working model is that it can be used as the basis for logical mutagenesis of either the restriction element or the peptide ligand. The results of such experiments, coupled with the data described in this report, will not identify absolute requirements for individual amino acids at the critical contact positions in the peptide, but rather might lead to a hierarchy of amino acid preferences. Such a ranking can be used to identify peptides from other protein sequences that can bind MHC class II molecules with high affinity.

Acknowledgements

We wish to thank Drs Steve Martin and Peter Bailey, National Institute for Medical Research, Mill Hill, for help with the circular dichroism

measurements, Dr Julia Bodmer and Steve Marsh for the anti-DR monoclonal antibodies and EBV-transformed B cell lines, Dr Roberto Accolla for the class II-negative B cell line, RJ 2.2.5, Dr M. Contreras, National Blood Transfusion Centre, Edgware, for providing human serum and peripheral blood lymphocytes, and Wendy Senior for help with preparation of the manuscript.

Abbreviations

CD	circular dichroism
EBV	Epstein – Barr virus
FITC	fluorescein isothiocyanate
HA	influenza haemagglutinin
IL-2	interleukin 2
LCB	long-chain biotin(yl)
MHC	major histocompatibility complex
PBMC	peripheral blood mononuclear leukocytes
[^{3}H]TdR	tritiated thymidine
TFE	trifluoroethanol
TPCK	tosylaminophenylalanylchloromethylketone

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0022-1767/91/1474-1292$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 147, 1292–1298, No. 4, August 15, 1991
Printed in U.S.A.

EFFECT OF NATURAL POLYMORPHISM AT RESIDUE 86 OF THE HLA-DR β CHAIN ON PEPTIDE BINDING

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Class I and class II MHC glycoproteins are highly polymorphic molecules that bind antigenic peptides and present them on cell surfaces for recognition by T lymphocytes. Even though MHC polymorphism has long been known to affect both peptide binding and recognition by the TCR, the role of individual amino acids of MHC proteins in these interactions is poorly understood. To examine the effect of a small number of amino acid residues on T cell stimulation, B lymphoblastoid cell lines homozygous for the closely related DR1 subtypes, Dw1 and Dw20, and the DR4 subtypes, Dw4 and Dw14, were compared for their ability to present an immunogenic influenza hemagglutinin peptide (HA307-319) to an Ag-specific, DR1,4-restricted T cell clone. B cell lines expressing DR1 Dw20 and DR4 Dw14 presented HA307-319 much less efficiently than DR1 Dw1 and DR4 Dw4 and bound a biotinylated analogue of the same peptide less well. Analysis of DRB1 gene sequences suggested that polymorphism at residue 86 had a major effect on peptide binding. Differences in binding of a set of HA307-319 analogues biotinylated at each residue to cells expressing DR1 Dw1 and DR1 Dw20 suggested that the polymorphism affected the interactions of many peptide residues with the class II molecule. In inhibition assays, DR1 Dw1 and DR4 Dw4 were shown to differ from DR1 Dw20 and DR4 Dw14 in their length requirements for peptide binding. Using a larger panel of homozygous B cell lines expressing many class II haplotypes, a Ser-309 substituted HA307-319 analogue was shown to bind to most B cell lines expressing Val-86 containing alleles (including DR1 Dw20 and DR4 Dw14) but failed to bind most B cell lines expressing Gly-86 alleles (including DR1 Dw1 and DR4 Dw4). The results indicated that polymorphism at residue 86 influenced the specificity and affinity of peptide binding and affected the conformation of peptide-DR protein complexes without completely eliminating T cell recognition.

T cells recognize peptides bound to class I and class II MHC proteins, and polymorphism in these proteins may influence T cell recognition either by affecting the formation of peptide-MHC protein complexes or by interfering with their recognition by the TCR (1–4). In the crystal structure of the class I protein, HLA-A2, most of the polymorphic residues either made direct contacts with bound peptides or were located close to the proposed peptide binding site at positions that might interact with a TCR (5). Crystallography has been used to examine directly the effect of polymorphism on the structure of the class I Ag combining site. Fifteen differences in primary structure between HLA-A2.1 and Aw68 affected the characteristics of individual specificity pockets but not the overall shape and dimensions of the Ag binding site (6). Homologies and conserved patterns of polymorphic residues between class I and class II proteins suggest that the binding site of class II MHC molecules has a similar structure and that polymorphism affects peptide binding and T cell recognition by analogous mechanisms (7).

The development of assays for peptide binding to affinity purified class II MHC proteins has permitted the effect of polymorphism on these interactions to be measured directly (8–10). Screening large numbers of peptides for binding to murine I-A and I-E class II proteins confirmed that the specificity was influenced by structural differences between MHC proteins. In addition, alleles encoded at the same locus were more similar in their structural requirements for peptide binding than different class II isotypes (11–13). Even though these studies demonstrated that polymorphism affected peptide binding, individual important residues in the class II molecules were not identified, primarily because a small number of alleles were compared and these differed from each other at multiple residues.

In this study, residue 86 of the HLA-DR β chain was shown to be critical for binding of an influenza hemagglutinin peptide. This was done by comparing structural requirements for binding to DR alleles differing at a small number of amino acid residues and by correlating HLA-DR amino acid sequence with specificity across many different DR types. Polymorphism at residue 86 appeared to affect the structure of the peptide-DR complex as a whole, rather than local interactions with an individual peptide residue.

Received for publication October 23, 1990.
Accepted for publication May 30, 1991.
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MATERIALS AND METHODS

Peptides. Peptides representing residues 307-319 of the influenza A/Texas/1/77 H3 hemagglutinin protein (HA307-319, P K Y V K Q N T L K L A T) and analogues modified as shown in Table I were synthesized and long chain biotinylated as previously described (14).

Cells. B cell lines were grown in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 10% FCS (Sigma Chemical Co., St. Louis, MO), penicillin, and streptomycin. The lines used to compare DR1

TABLE I

Peptides used in this study

Name	Amino Terminal Group[a]	Sequence[b]												
Truncated and substituted analogues of HA307-319														
HA307-319	NH_2	P	K	Y	V	K	Q	N	T	L	K	L	A	T
Ac308-319	AcNH	-	-	-	-	-	-	-	-	-	-	-	-	-
308-319	NH_2	-	-	-	-	-	-	-	-	-	-	-	-	-
Ac309-319	AcNH		-	-	-	-	-	-	-	-	-	-	-	-
309-319	NH_2		-	-	-	-	-	-	-	-	-	-	-	-
Ac310-319	AcNH			-	-	-	-	-	-	-	-	-	-	-
310-319	NH_2			-	-	-	-	-	-	-	-	-	-	-
Ser-309	NH_2	-	-	S	-	-	-	-	-	-	-	-	-	-
Position of Biotinylation														
Biotinylated analogues of HA307-319														
		P	K	Y	V	K	Q	N	T	L	K	L	A	T
Amino terminus (HA LCB-N)	LCB-NH	-	-	-	-	-	-	-	-	-	-	-	-	-
Amino terminus	LCB-NH	-	R	-	-	R	-	-	-	-	R	-	-	-
Residue 307	AcNH	*B*	R	-	-	R	-	-	-	-	R	-	-	-
Residue 308	AcNH	-	*B*	-	-	R	-	-	-	-	R	-	-	-
Residue 309	AcNH	-	R	*B*	-	R	-	-	-	-	R	-	-	-
Residue 310	AcNH	-	R	-	*B*	R	-	-	-	-	R	-	-	-
Residue 311	AcNH	-	R	-	-	*B*	-	-	-	-	R	-	-	-
...								...						
Residue 318	AcNH	-	R	-	-	R	-	-	-	-	R	-	*B*	-
Residue 319	AcNH	-	R	-	-	R	-	-	-	-	R	-	-	*B*

[a] Functional groups at the amino terminus are abbreviated as follows: LCB, long chain biotinyl (6-(biotinamido)hexanoyl); Ac, acetyl; NH_2, free α amino group.

[b] Sequences are shown in standard single letter code, except that *B* denotes long chain biotinylated lysine. Dashes indicate identity with HA307-319.

and DR4 subtypes are shown in Table II, and a larger panel covering a range of haplotypes is shown in Table III. The T cell clone, HA1.7, was cultured in the presence of feeder cells, antigen, and IL-2 as described previously (15).

Binding of peptides to B lymphoblastoid cell lines. The assay was performed as described previously (16). Briefly, living B lymphoblastoid cells, homozygous for HLA DR proteins, were incubated in duplicate wells with HA LCB-NH or other biotinylated analogues at 37°C in 200 μM PBS/2.5% FCS. After two washes in PBS/BSA, the cells were stained in the cold with fluoresceinated avidin D (Vector Laboratories, Inc., Burlingame, CA) and analyzed by flow cytometry to determine relative amounts of bound biotinylated peptide. Dead cells were detected using propidium iodide (Sigma) and excluded from the analysis. HLA-DR expression was measured using saturating amounts of the fluoresceinated anti-DR monoclonal antibody, L243 (Becton Dickinson, Mountain View, CA). To control for slight variations in DR expression in quantitative comparisons of different cell lines for peptide binding, the mean fluorescence obtained with biotinylated peptide and streptavidin was divided by the fluorescence obtained with L243 and the ratio expressed as a percentage. Inhibition was measured by incubating the cells for 6 h with 25 or 50 μM HA LCB-NH in the presence of unbiotinylated peptides. The fluorescence intensities of duplicate samples usually were within 5% of the mean.

T cell proliferation assay. B lymphoblastoid cells (1×10^6/2 ml/well) were irradiated (5000 rad), incubated overnight at 37°C with HA307-319 or HA LCB-NH and washed twice with PBS/BSA. Aliquots of cells incubated with biotinylated peptide were removed and assayed for peptide binding. The remainder was incubated for 3 days in triplicate cultures at 2×10^4/well with an equal number of HA1.7 cloned T cells specific for HA307-319 (15). Tritiated thymidine (1 μCi/well) was added during the last 16 h of culture. Cells were harvested onto glass fiber filters, and thymidine incorporation was measured by liquid scintillation counting. Background proliferation in the absence of peptide was subtracted.

RESULTS

Presentation of influenza hemagglutinin peptide to T cells by different HLA-DR alleles. To examine the effect of structural differences between selected DR1 and DR4 subtypes (Table II) on T cell recognition, homozygous B lymphoblastoid cell lines expressing these subtypes were incubated with HA307-319, washed, and assayed for their ability to stimulate proliferation of the HLA-DR restricted, hemagglutinin-specific Th cell clone, HA1.7 (15). DR1 Dw1-homozygous cells induced a strong proliferative response when pulsed with as little as 20 nM peptide (Fig. 1*A*). In contrast, when the line expressing DR1 Dw20 was used, about 10^4 times higher peptide concentrations were required to stimulate similar levels of T cell proliferation (Fig. 1*A*). A difference also was observed when B cell lines expressing the DR4 subtypes were compared (Fig. 1*B*). Cells expressing DR4 Dw4 presented the HA peptide almost as well as those expressing DR1 Dw1, but presentation by cells expressing DR4 Dw14 was much less efficient. Similar numbers of DR molecules were expressed on all cell lines (data not shown), indicating that the observed differences were not due to differences in DR expression. Therefore, DR1 Dw20 and DR4 Dw14 molecules on B lymphoblastoid cells required much higher peptide concentrations for equivalent T cell proliferation than DR1 Dw1 or DR4 Dw4 molecules, indicating that the differences between the subtypes of DR1 and DR4 strongly influenced Ag recognition.

Binding of biotinylated HA307-319 to DR1 and DR4 molecules on B cell surfaces. An analogue of the HA307-319 peptide containing a long chain biotinyl group at the amino terminus (HA LCB-NH, Table I) was used to examine whether the DR molecules expressed on the four B cell lines differed in their ability to bind the peptide. Control experiments showed that biotinylation did not affect the ability of the peptide to stimulate the T cell

TABLE II

Predicted DRB1 chain sequences of DR1 and DR4 molecules analyzed in this study

HLA-DR Specificity	Cell Lines Used	1 10 20 30 40
DR1 Dw1	IBW4, MAJA, METTE	GDTRPRFLWQ LKFECHFFNG TERVRLLERC IYNQEESVRF
DR1 Dw20	LWAGS, PMG075	---------- ---------- ---------- ----------
DR4 Dw4	PRIESS	--------E- V-H------- -----F-D-Y F-H---Y---
DR4 Dw14	BIN40	--------E- V-H------- -----F-D-Y F-H---Y---

	50 60 70 80 90
DR1 Dw1	DSDVGEYRAV TELGRPDAEY WNSQKDLLEQ RRAAVDTYCR HNYGVGESFT VQRR
DR1 Dw20	---------- ---------- ---------- ---------- ----AV---- ----
DR4 Dw4	---------- ---------- ---------- K--------- -----G---- ----
DR4 Dw14	---------- ---------- ---------- R--------- -----V---- ----

TABLE III

Correlation between valine at residue 86 of DRB1 chain and binding of Ser-309 analogue[a]

HLA-DR type	Cell Line	Inhibition by HA307-319	Inhibition by Ser-309	Ratio	
		Gly-86 DRB1 alleles			
DR1 Dw1	IBW4	86	0	0	(Y > S)
	MLV	86	0	0	(Y > S)
DR4 Dw4	PRIESS	84	4	0.05	(Y > S)
	BSM	99	10	0.1	(Y > S)
DR16 Dw21	AZH	88	0	0	(Y > S)
	WT18	86	0	0	(Y > S)
DR4 Dw15	SJAH	82	4.2	0.05	(Y > S)
DR7 Dw7	MANN	79	14	0.18	(Y > S)
	MOU	99	11	0.11	(Y > S)
DR8 Dw8.2	OLL	86	31	0.36	(Y > S)
DR11 Dw5	BM21	96	42	0.44	(Y > S)
DR14 Dw16	AZL	75	0	0	(Y > S)
DR16 Dw22	REM	88	0	0	(Y > S)
		Val-86 DRB1 alleles			
DR1 Dw20	LWAGS	93	100	1.08	(Y = S)
DR4 Dw14	BIN40	65	43	0.66	(Y = S)
DR4 Dw10	AL10	96	54	0.56	(Y = S)
DR11 DwJVM	JVM	98	92	0.94	(Y = S)
DR14 Dw9	WT52	68	62	0.91	(Y = S)
DR15 Dw2	PGF	86	0	0	(Y > S)
DR17 Dw3	WT20	94	65	0.69	(Y = S)

[a] Each of the cell lines was analyzed for inhibition of binding of HA LCB-NH by the two unbiotinylated peptides as described in *Materials and Methods*. Binding of HA LCB-NH to these cell lines previously has been shown to be specific for DR using mAb (16). The percentage competition is shown for each cell line in the presence of 1 mg/ml inhibitor and 50 μM HA LCB-NH. Y > S indicates that the percent inhibition with the tyrosine substituted peptide is more than twice that seen with the serine substituted peptide. Y = S indicates that the percent inhibition with the tyrosine substituted peptide is less than twice that seen with the serine substituted peptide.

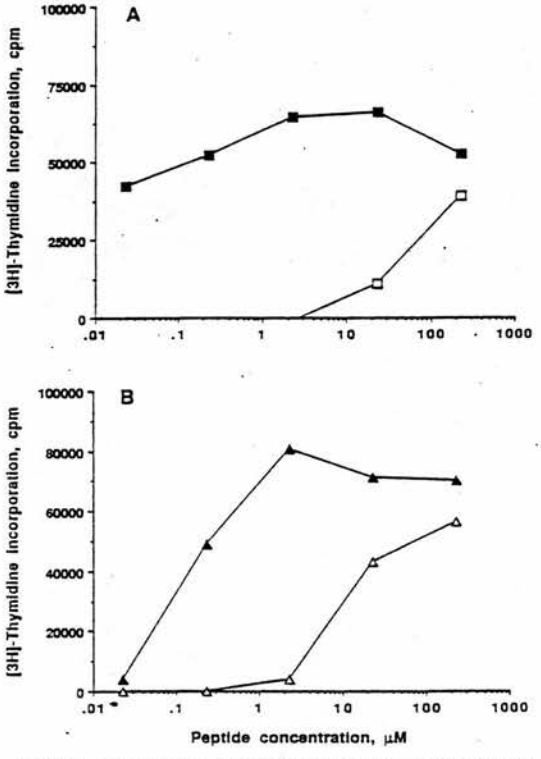

Figure 1. Presentation of HA307-319 to cloned T cells by B lymphoblastoid cells expressing DR1 and DR4 subtypes. *A*, Comparison of cell lines expressing the DR1 subtypes. Dw1 (*closed squares*) and Dw20 (*open squares*). *B*, Comparison of lines expressing the DR4 subtypes. Dw4 (*closed triangles*) and Dw14 (*open triangles*). Irradiated B lymphoblastoid cell lines were incubated overnight at 37°C with the indicated concentrations of HA307-319 and washed before adding the T cell clone, HA1.7. A background of approximately 4000 cpm were subtracted from each data point.

clone when presented by these B cell lines (data not shown). Each cell line was incubated with HA LCB-NH at 37°C, stained with fluoresceinated avidin, and analyzed by flow cytometry. This assay previously has been shown to correlate well with binding to purified DR molecules (10). At any one peptide concentration, cells expressing DR1 Dw1 gave the highest fluorescence, followed by DR4 Dw4 homozygous cells. DR1 Dw20 and DR4 Dw14 homozygous cells bound the peptide much less well and required very high peptide concentrations for a distinct fluorescent signal to be observed (Fig. 2, *A* and *B*). Thus, the alleles showed the same order of reactivity in the cell surface binding and proliferation assays, although the differences in Ag presentation between the B cell lines were larger than the differences in peptide binding. On all four cell lines, the specificity of binding previously has been demonstrated using anti-class II mAb (16). Therefore, at least some of the differences in presentation of the HA peptide between the different DR1 and DR4 subtypes could be attributed to differences in their ability to form complexes with the peptide on the cell surface.

Mapping of differences in binding and T cell recognition between subtypes to residue 86 of DRβ1 chain. The amino acid sequences of the DR1 and DR4 subtypes were compared to identify residues that might cause the observed differences in peptide binding (Table II). DR1 Dw1 and DR1 Dw20 β chains differed from each other only at residues 85 and 86, indicating that one or both of these sequence differences influenced peptide binding. The DR4 subtypes differed from each other at residues 71 and 86, but both DR4 subtypes differed from DR1 at multiple positions. The high binding alleles, DR1 Dw1 and DR4 Dw4, shared a glycine residue at position 86, whereas the low binding alleles, DR1 Dw20 and DR4 Dw14, shared a valine at this position. This showed that polymorphism at residue 86 was sufficient to explain the differences in binding of the HA peptide to the DR1 and DR4 subtypes.

Effect of position of biotinylation on peptide binding to DR1 Dw20 homozygous cells. A set of HA307-319 analogues biotinylated at each position (Table I) was used to examine whether polymorphism at residue 86 (and/or residue 85) affected the structural requirements for peptide binding to DR1 subtypes. Binding of these analogues to DR1 Dw1 homozygous cells previously has been shown to depend markedly on the position of biotinylation (14). None of these analogues bound to mutant B cells lacking DR and DQ expression, and binding to DR1 Dw1 homozygous B cells was inhibited by anti-DR mAb (14) (data not shown). These studies also have provided evidence that differences in fluorescence observed with these analogues were due to biotinylation interfering with peptide

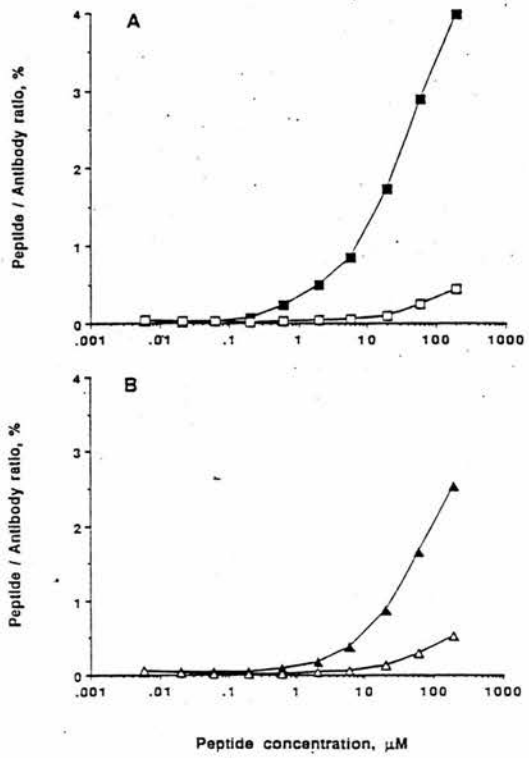

Figure 2. Binding of HA LCB-NH to B-lymphoblastoid cells expressing DR1 and DR4 subtypes. *A*, Cells expressing the DR1 subtypes, Dw1 (*closed squares*) and Dw20 (*open squares*) were incubated with HA LCB-NH as described in the legend to Figure 1, stained with fluoresceinated avidin, and analyzed by flow cytometry. *B*, Cells expressing the DR4 subtypes, Dw4 (*closed triangles*) and Dw14 (*open triangles*) were analyzed for binding. To correct for slight variations in DR expression between lines, the mean fluorescence due to peptide binding was divided by the fluorescence obtained with a fluoresceinated anti-DR antibody and expressed as a percentage.

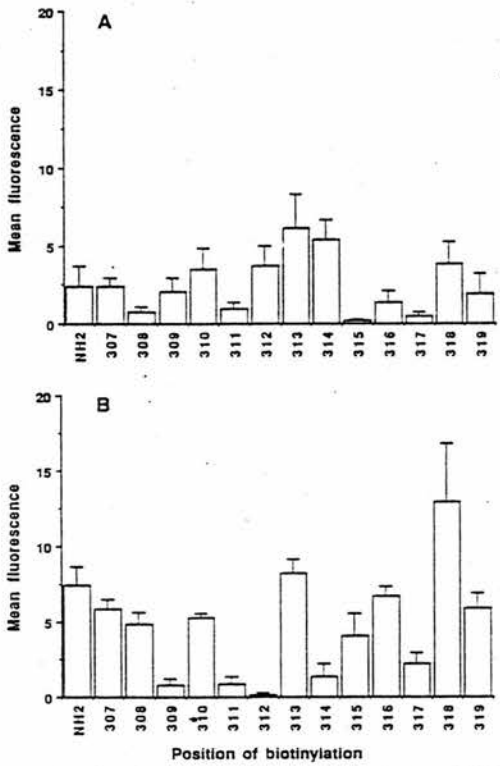

Figure 3. Effect of the position of biotinylation on binding of HA307-319 analogues to cell surface DR1 Dw1 and Dw20 molecules. Cells expressing DR1 Dw20 (*A*) or DR1 Dw1 (*B*) were incubated with a set of HA307-319 analogues (50 μM) substituted at each residue with long chain biotinylated lysine (see Table I). The data in *B* have been published previously (14). The bar labeled NH2 refers to the analogue biotinylated on the α amino group.

binding to DR1 Dw1 molecules, rather than effects of biotinylation on the conformational propensities of the peptides or their susceptibility to proteases (14). The variations in fluorescence therefore appeared to provide information on the relative ability of each residue to tolerate substitution with a bulky side chain and hence on the conformation of the bound peptide.

When each analogue was examined for its ability to give a fluorescent signal on cells expressing DR1 Dw20 (Fig. 3*A*), the fluorescence intensities were, on average, lower than on cells expressing DR1 Dw1 (shown for comparison in Fig. 3*B*). In addition, the dependence of the fluorescent signal on the position of biotinylation was different on cells expressing DR1 Dw20 than the pattern of fluorescence previously seen on DR1 Dw1. Biotinylation at residues 309, 312, and 314, which greatly reduced or eliminated the fluorescent signal on DR1 Dw1 expressing cells, did not drastically affect binding to DR1 Dw20 cells. In contrast, biotinylation at residues 308, 315, and 316 reduced the fluorescence on cells expressing DR1 Dw20 more than on those expressing DR1 Dw1. Similar results were obtained with two other cell lines expressing DR1 Dw1 and one other line expressing DR1 Dw20 (data not shown). Taken together, the data suggested that the contacts of many residues of the HA307-319 peptide were affected by the differences between the DR1 subtypes.

Length requirements for peptide binding to DR1 and DR4 subtypes. To confirm that subtype differences altered the way in which the peptide bound DR1 and DR4 molecules, truncated analogues of the HA307-319 peptide were analyzed for their ability to inhibit binding of the biotinylated peptide to each of the cell lines (Table I; Fig. 4). Removal of residues 307 and 308 from the amino terminus of the HA peptide had no effect on its ability to interact with cell surface DR1 Dw1 and DR4 Dw4 molecules in the competition assay, but removal of residue 309 resulted in complete loss of binding (Fig. 4, *A* and *C*). A different pattern was observed when cell lines expressing DR1 Dw20 or DR4 Dw14 were examined. On these cells, removal of residues 307, 308, and 309 resulted in a gradual loss of inhibition (Fig. 4, *B* and *D*). Acetylation of the α amino group in these peptides had little effect on binding, indicating that the results were not influenced by effects of the amino terminal charge on the interaction

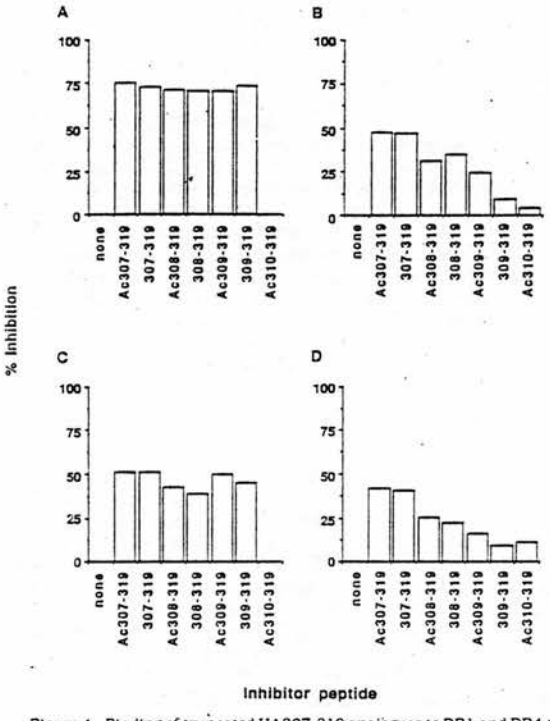

Figure 4. Binding of truncated HA307-319 analogues to DR1 and DR4 Dw subtypes. Cells expressing DR1 Dw1 (*A*), Dw20 (*B*), DR4 Dw4 (*C*), or DR4 Dw14 (*D*) were assayed for binding of the truncated peptides in a competition assay. Each cell line was incubated with HA307-319 or truncated analogues (200 μM final concentration) and 25 μM HA LCB-NH and stained with streptavidin. Results are expressed as percent inhibition of the fluorescent signal compared to the signal obtained in the absence of unbiotinylated peptide.

with the DR molecule. These results showed differences in length requirements for peptide binding to DR1 Dw1 and DR4 Dw4 on the one hand and DR1 Dw20 and DR4 Dw14 on the other and suggested that the way in which the peptide bound was altered by polymorphism at residue 86 of the DR β chain.

Correlation between binding of substituted peptide and residue 86 of DRβ1 chain. To confirm the validity of the differences in peptide binding between DR1 Dw1 and Dw20 observed using biotinylated peptides, residue 309 was examined in greater detail. This residue was chosen for further investigation because previous experiments have shown that it is critical for binding DR1Dw1 and DR4Dw4 (14, 17) (unpublished observations). Deletion or substitution of tyrosine 309 with amino acids with either small or polar side chains resulted in analogs that were unable to bind DR1Dw1 or DR4Dw4. In addition, all residues other than tyrosine 309 and lysine 316 in this determinant can be substituted with alanine without any significant loss in apparent affinity for DR1Dw1. Biotinylation at this residue also greatly reduced the fluorescent signal on cells expressing DR1 Dw1 but had little effect on fluorescence when DR1 Dw20 expressing cells were used (Fig. 3). The cell lines expressing DR1 and DR4 subtypes therefore were examined for binding of an HA307-319 analogue containing a serine substitution at residue 309 (Ser-309, Table I). The substitution greatly reduced peptide binding to DR1 Dw1 and DR4 Dw4 but had no effect on binding to cells expressing DR1 Dw20 or DR4 Dw14 (Table III). This confirmed that the importance of residue 309 of the peptide for the formation of cell surface peptide-DR complexes was influenced by polymorphism at residue 86 of the DR β chain.

The specificity of peptide binding was influenced in similar ways by subtype differences in both DR1 and DR4 alleles, even though DR1 and DR4 differ from each other at multiple positions. This suggested that effects of polymorphism at residue 86 on peptide binding might be detected in haplotypes other than DR1 and DR4. Therefore, a larger panel of B cell lines, homozygous for HLA DR proteins and representing many of the defined polymorphisms among DRB1 first domain sequences (18), was analyzed for binding of the HA307-319 peptide and the Ser-309 analogue. A striking correlation was observed between binding of the serine substituted peptide and residue 86 of the DR β chain (Table III). All of the 13 B cell lines expressing 9 different DRB1 genes coding for Gly at residue 86 bound the natural HA307-319 peptide but not the Ser-309 analogue. On these cell lines, residue 309 of the peptide appeared to make an important contact with the DR molecule. In contrast, the substitution had no effect on binding to most cell lines expressing Val-86 containing DRB1 genes (6/7 lines, representing 6/7 haplotypes), indicating that residue 309 was not important for binding to these cell lines. Cell lines expressing subtypes of DR4 and DR6 differed in their reactivity with the Ser-309 peptide despite sharing the DRw53 and DRw52 genes, respectively. This confirmed that most of the biotinylated peptide bound to the more abundant DRαβ1 molecule on these cell lines, even though binding of HA LCB-NH to DRw52 molecules has been observed using transfected L cells[2] (R. Busch, unpublished observations). However, on cells expressing the DR2 Dw2 gene (Val-86), the substituted peptide failed to compete, and on DR11 Dw5 homozygous cells (Gly-86), the substitution had no effect. On these cells, the HA peptide therefore may either bind to the DRαβ1 molecule in a different way than to most other alleles, or the fluorescent signal may be due to binding to products of the second DR β chain gene. Nevertheless, the significant correlation between the ability of the Ser-309 analogue to inhibit binding and the presence of valine at residue 86 of the DRβ1 chain strongly suggested a role for residue 86 in determining the specificity of peptide binding to many different DRαβ1 molecules.

DISCUSSION

Polymorphism at residue 86 of the DR β chain has been shown in this report to influence recognition of an immunogenic influenza virus HA peptide (residues 307-319) by Ag-specific, DR1.4-restricted cloned T cells. This was at least partly due to the effect of this polymorphism on the amount of peptide binding to cell surface DR molecules. Structural variation at residue 86 correlated with differences in the fine specificity of binding to closely related DR1 and DR4 subtypes. This was shown by dif-

[2] O'Hehir, R. E., R. Busch, J. B. Rothbard, and J. R. Lamb. An in vitro model of peptide-mediated hyposensitization of the human T cell response to Dermatophagoides spp. (house dust mite). *Submitted for publication.*

ferences in binding of monosubstituted analogues and peptides truncated at the amino terminus to B cell lines homozygous for these DR alleles. Sensitivity of binding to a substitution at residue 309 of the peptide correlated with the presence of Gly at residue 86 of the DR β chain in many different haplotypes. The polymorphism appeared to affect the contacts of multiple residues throughout the entire peptide sequence with the DR molecules, as suggested by differential effects of biotinylation on binding to B cell lines expressing DR1 Dw1 and Dw20. Together, these observations showed that residue 86 affected the specificity of peptide binding to DR molecules, possibly as a consequence of altering the shape of the Ag combining site and consequently the contacts made with bound peptides.

Even though residue 86 is not highly polymorphic, being either a glycine or a valine in all reported HLA-DR β chain sequences, and is located outside the three regions of greatest allelic variability (18), a growing body of evidence indicates that polymorphism at this position affects Ag recognition by T cells. In humans, DR subtypes differing at residue 86 or at residues 85 and 86 are distinguishable by alloreactive T cells, and pairs of alleles differing at these residues have been identified for many different DR types (18, 19, 20). The homologous region of murine class II molecules also has been shown to be important for T cell recognition. Site-directed mutagenesis of murine I-A^k β chain genes showed that substitutions at residues 85, 86, and 88 affected T cell recognition (21). Susceptibility to type II collagen-induced arthritis in DBA/1 mice has been mapped to residues 85-89 of the I-A^q β chain (22). The data reported here raise the possibility that polymorphism at residue 86 of class II β chains may play a crucial role in each of these systems by influencing the repertoire of bound peptides. However, our results do not rule out the possibility that residue 86 may additionally interact with the TCR, as has been suggested for the homologous region of the I-A^k β chain (21).

According to current models of the Ag combining site of MHC class II proteins, based on similarities between class I and class II proteins and the known crystal structure of the class I molecule, HLA-A2, residue 86 is predicted to be located at the carboxyl-terminus of the β chain helical region that forms one wall of the Ag binding site and to point into the site (7). The polymorphism at this residue not only affected local interactions between the DR molecule and the amino terminus of the peptide, but appeared to alter the structure of the entire peptide-DR complex. This contrasts with a substitution affecting a single pocket in the binding site, as was observed in our previous experiments studying the possible interactions between residue 71 of the β chain and 312 of the HA peptide (23). In these experiments, T cell recognition could be reversed by changing the residue at 312 in the peptide to complement the charge characteristics of residue 71 of the DR protein. Effects of the substitution on other residues in the peptide were not observed. In contrast, modifications at residue 86 of the β chain resulted in a more global effect than a modification of a single pocket in the binding site. The strongest evidence supporting this hypothesis was the manifold differences observed in fluorescent patterns associated with the set of peptides biotinylated at each position when assayed on DR1Dw1 compared with DR1Dw20. Also the majority of alleles with glysine at 86 required an amino acid at 309 with a bulky hydrocarbon sidechain. Substitution with small or polar amino acids (i.e., serine) obviated all binding. In contrast, alleles with valine at 86 bound the peptide with serine at 309, and also those with tyrosine at this position. This lack of complementation indicates that the results are not due to a simple interaction between residue 86 of the β chain and 309 in the peptide.

The results could be explained in either of two ways. A direct contact between residue 86 and the HA peptide might alter the conformation of the remainder of the peptide. Alternatively, residue 86 might affect the interaction of the β chain helix with nearby α chain residues, altering the geometry of the Ag combining site. The two mechanisms could not be distinguished by our results and may not be mutually exclusive.

None of the DR1 and DR4 subtypes studied failed to present the peptide to cloned T cells, even though the T cell clone used was exquisitely sensitive to structural changes in the peptide and the DR protein (14, 15, 23). This suggested that the differences in intermolecular contacts observed using biotinylated HA analogues were not a result of drastic changes in the way the peptide bound, but rather the consequence of subtle adjustments in the structure of the complex owing to the polymorphism. The correlation between Val-86 and binding of the Ser-309 analogue in many different DR haplotypes also suggested that most DR alleles did not differ drastically in the way they bound the peptide. These ideas are consistent with our earlier observations on differences in binding and T cell reactivity between DR4 Dw4 and Dw13 (JHF) on the one hand and DR4 Dw10 on the other (23). The former two are Gly-86 alleles and, when tested with the biotinylated HA peptides, revealed a similar conformation of the bound peptide as that shown in Figure 3*B*. The latter allele contains Val at residue 86 and bound the HA peptide in a different way as measured by differences in the pattern of biotinylation. Nevertheless, these conformational differences were not drastic enough to prevent HA1.7 from recognizing an HA307-319 analogue when presented by this restriction element. In each case, the differences in sensitivity to biotinylating each residue on these alleles could be explained by minimal deviations from a common conformation (23). Crystallographic studies of defined peptide-MHC class II complexes will be required to analyze the precise nature and extent of the conformational differences.

Regardless of the exact way in which the Ag binding site is altered by polymorphism at residue 86, our results indicate that effects on the specificity of peptide binding can be mapped to individual residues of class II proteins. A peptide that has been shown to interact with many different DR alleles has been converted by a single amino acid change into one that binds a much smaller subset of alleles. This strongly suggests a role for polymorphism at residue 86 in altering and limiting the spectrum of peptides bound to individual DR alleles. In addition, the observation raises the possibility that allele specific peptides can be designed rationally, starting from degenerate ones, by detailed empirical analyses of the effects of class II polymorphism on the fine specificity of binding.

Acknowledgments. We are grateful to S. G. E. Marsh.

J. G. Bodmer (Imperial Cancer Research Fund, London, England), G. Ruberti and C. G. Fathman (Stanford University) for providing B cell lines, to Vineeta Bal (Hammersmith Hospital, London) for help with T cell proliferation assays, and to K. Howland and C. Fenton (Imperial Cancer Research Fund) for peptide synthesis and purification.

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Mapping T cell recognition: the identification of a T cell receptor residue critical to the specific interaction with an influenza hemagglutinin peptide

The fine specificity of T cell receptor (TCR) interaction with the influenza hemagglutinin peptide HA 307–319 in the context of the DR1 (DRA, DRB1*0101) and DR4 (DRA, DRB1*0404) was studied in two human T cell clones (HA1.7 and Cl-1) derived from different individuals. Sequencing of amplified TCR transcripts revealed that these two clones express highly related TCRα chains, with a conserved junctional motif, but very different TCRβ chains. Modeling studies led to the prediction that the conserved glutamic acid residue in the TCRα chain could interact with the lysine at position 316 in the peptide, a known TCR contact residue. HA1.7 TCR-CD3ζ chimeric constructs were expressed in the rat basophil line (RBL) and shown to confer specific antigen recognition. In two TCRα chain mutants, with the conserved glutamic acid residue altered to alanine and lysine, respectively, peptide recognition was lost. Specific recognition was not rescued by altered peptide ligands. Furthermore, Jurkat derivatives expressing the related Jurkat TCRα chain paired with the HA 1.7 TCRβ chain did not recognize the HA 307–319 /DR1 complex. These data provide evidence for the critical interaction of a TCR residue with antigenic peptide.

1 Introduction

T cells recognize antigen as short peptides presented by MHC molecules [1, 2], using the highly variable, clonally distributed T cell receptor (TCR). Elucidation of the crystal structures of the DR1 (DRA/DRB1*0101) class II molecule [3] and several class I molecules [4, 5], have confirmed that antigenic peptides are bound in a groove of the MHC molecule, with certain amino acid side chains particularly available for interaction with the antigen-specific TCR. While the three-dimensional structure of the TCR is still unknown, comparative modeling on the basis of homology with the immunoglobulins has suggested the presence of three highly variable complementarity-determining regions (CDR1, 2 and 3) which are thought to form the specific antigen binding site [6, 7]. Many studies have suggested that particular MHC/peptide complexes are recognized by a limited number of TCRα or TCRβ gene segments in both murine and human T cells [8–12]. Furthermore, much evidence exists for the critical nature of interactions between the proposed CDR3 regions of the TCR chains and antigenic peptide, with many studies showing conservation of TCRα or TCRβ, or both, CDR3 amino acid sequences, when a particular peptide/MHC complex is recognized [12–14]. Most compellingly, in mice transgenic for either the TCRα or TCRβ chain of a TCR specific for moth cytochrome c, the alteration of a single amino acid residue in the peptide resulted in specific TCR showing reciprocal single-residue changes in the CDR3 regions of the nontransgenic chain *in vivo* [15].

The human $CD4^+$ T cell response to the influenza hemagglutinin (HA) peptide 307–319 (PKYVKQNTLKLAT) in the context of DR1 has been extensively studied [16–18]. Note that two alternative nomenclatures exist for this protein, such that the same peptide is known as 306–318 in some publications [3]. Functional studies have implicated several residues in HA 307–319 as important TCR contact residues: these are residues 308K, 311K, 316K and 319T ([17, 19, 20], and LRW and JRL, unpublished). In general, peptide analogs with substitutions at these positions are not recognized by HA/DR1 specific T cell clones, although these peptides still bind DR1 [17]. At position 316 of the HA peptide, most substitutions create a non-stimulatory peptide. Two exceptions are the HA307–319 peptides with substitutions 316R and 316C, which stimulate the HA1.7 clone, but only at far higher concentrations than that required for native HA307–319 (LRW, and JRL, unpublished observations). For another human clone specific for HA307–319/DR1, Cl-1, several altered peptides have been shown to act as potent antagonists at low concentrations (for example HA307–319–316L) [19]. The recent elucidation of the crystal structure of the HA307–319/DR1 complex has confirmed that the residues 308K, 311K, 313N, 316K and 319T do indeed project away from the MHC binding groove, and are available for interaction with the TCR [3]. However, residues in the TCR of HA307–319/DR1-specific T cell clones which interact directly with these amino acids in the peptide have not been identified.

Here, we analyze the TCR of two HA307–319/DR1-specific T cell clones, which also recognize peptide presented by DR4 (DRB1*0404) class II molecules. The

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Abbreviations: **HA:** Hemagglutinin **RBL:** Rat basophil line **An PCR:** Anchored polymerase chain reaction

Key words: T cell receptor / Hemagglutinin / HLA-DR1 / Influenza

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development of new methods for modeling the T cell receptor has allowed the construction of a plausible model of the ternary complex. Consideration of this model, in combination with mutagenesis *in vitro* studies, have led to the identification of a residue in the TCRα chain junctional region that is essential for the interaction of the HA1.7 with its ligand.

2 Materials and methods

2.1 T cell clones and cell lines

The Cl-1 clone was a kind gift of Dr. H. Grey (Cytel, San Diego) [18]. The HA1.7 and Cl-1 T cell clones were maintained culture by addition of irradiated (2500 rad) feeder cells and peptide every 7 days, and rIL-2 (10 ng/ml, Eurocetus, GB) every 3–4 days as described [16]. Prior to use in proliferation assays, the clones were rested for 6–7 days after the last addition of feeder cells and antigen. Irradiated PBMC from donors of DR1(DRB1*0101) or DR4 (DRB1*0404) haplotype were used as a source of APC for the T cell clones. The mutant TCR-negative Jurkat cell line JRT3T3.5, which lacks a full-length TCRβ mRNA, [21], was a gift of Prof. A. Weiss, (University of California, San Fransisco). JRT3T3.5, and the HA1.7 TCR-transfectants derived from it, including the clone CH7C17, were cultured as described [22]. The L cell transfectant LDR1 (DRB1*0101 positive) was a gift from Dr. J. Bodmer, Imperial Cancer Research Fund, London. CTLL, a murine IL-2-dependent T cell line [23], was used to assay IL-2 release from Jurkat derived transfectants, as described [22]. The rat basophil line RBL-2H3 was used for transfections of TCR-CD3ζ chimeric constructs [24]. The EBV-transformed B cell line HOM-2 (DRB1*0101 positive) was used as APC in the RBL stimulation assays.

2.2 Peptides, oligonucleotides and antibodies

The hemagglutinin peptide HA 307–319 (PKYVKQNTLKLAT), mutant HA peptides with altered residues at position 316, as well as the control DR1 binding matrix peptide MAT 19–31 (PLKAEIAQRLEDV), were synthesized by the peptide synthesis laboratory, Imperial Cancer Research Fund. Oligonucleotides were synthesized by Dr. I. Goldsmith, Imperial Cancer Research Fund. The JOVI-1 (TCR Cβ specific) and JOVI-3 (TCRVβ3 specific) antibodies were as described [25]. The pan anti-TCRα antibody αF1 was purchased from T cell Diagnostics, (Eurogenetics, GB). The anti-CD3 antibody UCHT1 was a kind gift from Prof. Peter Beverley, Imperial Cancer Research Fund. For cell staining, antibodies were used as direct fluorescein isothiocyanate (FITC) conjugates, or cells were counterstained with FITC-conjugated $F(ab')_2$ goat anti-mouse IgG, (Sigma, GB).

2.3 T cell clone proliferation assays

Cloned HA1.7 and Cl-1 cells (10^4) were cultured in the presence of 2.5×10^4 irradiated PBMC of the DR type shown and HA peptide (dose range 0–3 μg/ml), in flat-bottom 96-well plates for 72 h. Cells were pulsed with [^{3}H] dThd; 1 μCi/well (Amersham) for the last 16–18 h of the assay. Proliferation as [^{3}H] dThd incorporation was determined by liquid scintillation spectroscopy.

2.4 Sequencing of TCR

The TCRα and β sequences of the HA 1.7 clone have been published [22]. The TCR sequences of Cl-1 were obtained by anchored PCR (An PCR) as described [26]. Briefly, 5 μg of total RNA was used in first-strand cDNA synthesis, using a dT(12–18) primer and AMV reverse transcriptase. After precipitation, the product was G-tailed using terminal deoxynucleotidyltransferase, and 5% of the tailed cDNA was then used as template in An PCR reactions. Primers, PCR conditions and M13mp18 cloning were as described [26]. M13 subclones (15–20) were sequenced by the chain-termination method for each of the TCRα and TCRβ chains [27]. Sequences were compared to those available in the EMBL data bank.

2.5 Modeling of the TCR-DR1-HA ternary complex

HA1.7 was modeled according to the method of Searle, S.J. and Rees, A.R. (in preparation). Briefly, sequences of naturally paired antibody VH and VL regions were aligned with paired TCR Vα and Vβ regions from which framework and complementarity-determining regions (CDR) were identified. To determine whether the TCR sequences were more similar to VH or VL sequences, each set of Vα and Vβ sequences were compared to both the VL and VH sets, using the similarity score method described in [28]. In addition, the structural environments of TCR residues were compared to those of equivalent antibody residues by calculating a normalized similarity score for each residue in the context of neighboring residues within a 5Å radius. The results of these analyses showed that both Vα and Vβ have greater similarity to VL domains than to VH domains.

Given this VL preference, both Vα and Vβ framework regions were modeled using the light chain dimer RB1 [29] and the CDR sequences were modeled using a modified version of the antibody modeling program CAMAL [30, 31]. The final model was energy-minimized using Discover (Biosym). Since there is, as yet, no TCR structure, the accuracy of this method was tested by generating a model of CD4, a member of the immunoglobulin superfamily more distantly related to antibodies than TCR. When this model was compared with the x-ray structure of CD4 [32], the CDR had the following backbone RMS deviations: CDRd and β1 (1.6Å), CDRα and β2 (2.4Å) and CDRα3 and β (1.3Å). This close agreement with the x-ray structure gave sufficient confidence to proceed to construction of the ternary complex.

The peptide-MHC complex was generated using the x-ray corrdinates of the DR1 [33] and a peptide-docking procedure in which the peptide was positioned in the MHC groove in an extended orientation with its known TCR contact residues solvent-exposed. This modeled complex was subsequently modified to reflect the peptide twist described in the x-ray structure of the DR1-HA307–319 complex [3].

To generate the ternary complex, the TCR and MHC-peptide complex were rotated onto each other in an orientation that maximized the contact between CDR3 regions and the peptide contact residues. Two possible orientations of the TCR were seen, differing by a rotation of 180 ° with respect to the MHC-peptide complex. However, only one of these (see Fig. 3) allowed acceptable contacts to be made between CDR3 residues and the known peptide contact residues. In this preferred orientation, CDR1 and 2 of the TCRα chain and CDR2 of the TCRβ chain are in contact with the MHC helices, while CDR1 of the TCRβ chain probably has little or no contact.

A test of this procedure was carried out using the TCR5C.C7 and the MHC class I complex, pigeon cytochrome c in the context of I-E^k described by Jorgensen et al. [15]. Construction of the ternary complex in this system brought all the known TCR and peptide contact residues to within less than 5Å (Searle, S.J. and Rees, A.R. unpublished).

2.6 Expression of HA1.7 TCR chains in Jurkat lines

The HA1.7 TCRαβ transfectant CH7C17 has been previously described [22]. The expression of the HA TCRβ chain with the Jurkat TCRα chain was achieved by co-transfection of the HA1.7 TCRβ construct HAβ-pJ6Ω (35 μg) with the selection vector pJ6Ωhygro (5 μg) carrying a hygromycin resistance gene into 1.5×10^7 JRT3T3.5 cells. Cells were electroporated on a Biorad Gene Pulser using 250 V and 960 μF, and allowed to recover for 10 min on ice. Cells were selected in hygromycin (Calbiochem) at 1 mg/ml 24 h after electroporation. Cells were analyzed 12–14 days later for TCRβ expression by flow cytometry on a Becton Dickinson FASCcan using FITC-conjugated JOVI-3 antibody, and positive clones were expanded.

2.7 TCR stimulation of Jurkat transfectants

T cells (5×10^4) were co-cultured with equal numbers of APC in the presence of native or altered HA peptides (dose range 0–30 μg/ml), anti-TCR antibodies (1 μg/ml) in the presence of PMA at 0.5 ng/ml, or PMA (0.5 ng/ml) and ionomycin (0.5 μg/ml), in flat-bottom 96-well plates for 48 h. Supernatants were harvested at 48 h and used to stimulate 10^4 CTLL cells/well for 36 h. Proliferation was measured by addition of 1 μCi/well [^{3}H]dThd for the last 6 h of culture, as described above.

2.8 TCR-CD3ζ chimeric constructs and rat basophil line transfections

The TCRVα-Cα and TCRVβ-Cβ sequences of the HA 1.7 TCR terminating at the membrane-proximal cysteine residue, were cloned into the pCDL-SRα construct the CD3ζ transmembrane and cytoplasmic sequences [24], a kind gift of Dr. T. Ottenhoff. Site-directed mutagenesis of the HA1.7 TCRα chain was performed using the oligonucleotide-directed method in M13mp19, according to the manufacturer's instructions (Amersham, GB). TCRα chain mutants containing alterations at the glutamic acid at position 94, E-A and E-K, were constructed, and cloned into the same chimeric constructs. All constructs were checked by sequencing of both strands. RBL-2H3 cells (1.5×10^7) were co-transfected with 10 μg HA1.7 TCRβ-ζ construct, 10 μg of HA1.7 TCRα-ζ construct (wild type or mutant), and 2 μg of the $pH_{\beta}APr$-1-Neo vector to confer neomycin resistance, with the electroporation conditions described above. Cells were selected in G418 at 1 mg/ml (Gibco, Life Technologies) and pools of transfectants were expanded. Cells were analyzed by flow cytometry 12 days after transfection for TCRα and TCRβ expression by αF1 and JOVI-3, respectively, and positive cells were sorted by JOVI-3 expression on the FACStar (Becton Dickinson).

2.9 RBL-2H3 stimulation assays

HOM-2 cells were loaded with peptide by culturing in the presence of wild-type or mutant peptides at the given concentrations for 36–48 h, before use as APC. RBL-2H3 TCR transfectants were cultured for 18 h at 3×10^4/well in 96-well flat-bottom plates in the presence of 0.2 μCi/well of [1,2-^{3}H] (N)-hydroxytryptamine binoxalate (5HT; NEN, Dupont). Peptide-loaded HOM2 cells were washed once and resuspended at a concentration of 3×10^6/ml in RPMI/2 mM glutamine/2 % FCS (assay medium). Labeled RBL transfectants were washed 3 times in warm assay medium, and then incubated for 1 h in the presence of 3×10^5 loaded APC, or other stimuli as shown, in 100 μl. The anti-TCR Vβ 3 antibody JOVI-3 was used at a final concentration of 5 μg/ml. An aliquot of the supernatant was removed for estimation of released serotonin as a measure of RBL stimulation. Cells were also lysed in 0.5 % Triton X-100. Radioactivity released into the supernatants and in the cell lysates was measured by liquid scintillation counting. The specific serotonin release was calculated as: (cpm released/total lysate cpm + cpm released) × 100.

3 Results

3.1 Proliferation of T cell clones to HA 307–319 on DR1 and cross-reactivity on DR4

Two human $CD4^+$ class II restricted T cell clones were compared for proliferation to the HA 307–319 peptide in the context of DR1 and DR4 expressing APC (Fig. 1). There was no significant difference in the response of HA1.7 to DR1 (DRB1*0101) or DR4 (DRB1*0404) APC and HA peptide, (Fig. 1a). However, Cl-1 showed much lower responses to HA in the context of DR4 than DR1 over the same dose range (Fig. 1b). both clones responded equally to IL-2 alone.

3.2 TCRα and β sequences of the two HA-specific clones

An PCR and sequencing of 15–20 amplified TCR transcripts from the Cl-1 T cell clone detected only one rearranged TCRα and one TCRβ chain. The nucleotide and predicted protein sequences of the Cl-1 TCRα and TCRβ chains spanning the VJα and VDJβ junctions are shown in

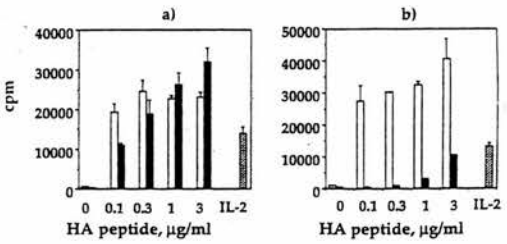


Figure 1. Recognition of HA 307–319/DR1 and HA 307–319/DR4 complexes by two T cell clones, a) HA1.7 and b) Cl-1. Clones were cultured with irradiated PBMC of the given MHC types as APC, and HA 307–319 peptide at concentrations shown, for 72 h, and proliferation measured by tritiated thymidine incorporation. Results are expressed as mean cpm ± SEM of triplicate cells. White bars, response using DR1 APC; black bars, response using DR4 APC; hatched bars, response to IL-2 only.

Fig. 2, with the equivalent sequences of the HA1.7 TCRα and TCRβ and the Jurkat TCRα for comparison. The Cl-1 TCRβ chain was previously designated Vβ13.2 [34] but under the new WHO nomenclature for TCR [35] has the name Vβ13.1 (TCRBV13S1) (Prof. J. Bell, personal communication). The HA1.7 clone expresses the Vβ3.1 segment (TCRBV3S1), and the two clones use different Dβ and Jβ genes. Furthermore, these two TCRβ chains show no conservation of either protein sequence or length across the presumed CDR3 region.

In contrast to the differences between the TCRβ chains, the TCRα chains utilized by these two clones are highly related, and show remarkable sequence conservation across the junctional region. The HA1.7 TCRα chain uses the Vα1.2 gene segment (TCRAV1S2A2), while the Cl-1 TCRα chain uses a polymorphic variant of Vα1.3 (TCRAV1S3), with two nucleotide changes which alter residues 57 and 58 such that they are the same as the equivalent residues in the Vα1.2 segment. In the published TCRAV1S3 gene segment, these residues are E-S while in the Cl-1 TCRα chain they are replaced by K-G, which are identical to the equivalent residues in the TCRAV1S2A2 segment. Furthermore, the clones have a conserved motif across the presumed CDR3 region, as defined by Chothia et al. [6]. Thus, both clones have the protein sequence V-S-E at the V/N/J junction, and this motif is followed by S or T, respectively, and then a helix-breaking residue, P or G, respectively. The highly related Jurkat TCRα chain uses the Vα1.2 segment (TCRAV1S2A2) and has the sequence V-S-D-L-E at the equivalent positions. The J genes of all three of the TCRα chains considered are different (Fig. 2).

3.3 Models of the TCR-HA-DR ternary complexes

Ternary complexes were generated in two orientations. In the preferred orientation (Fig. 3a,b) the five known peptide-contact residues (308K, 311K, 313N and 316K and 319T) are all in contact with various CDR residues. One of the peptide residues (316K) is seen to be in contact with an α-chain residue, 94E (see Fig. 2). This type of salt bridge interaction has also been implicated in the study of Jorgensen et al. [15]. In the second orientation (Fig. 3c), 316K is seen to be outside the contact influence of the TCR CDR, so this is therefore a less likely structure for the ternary complex.

3.4 Expression of the HA1.7 TCR chains in RBL and JRT3T3.5

Previous studies with the HA1.7 and Cl-1 T cell clones have implicated the 316K as an important residue in T cell recognition [17–19]. In order to test the prediction of the modeling studies that the conserved glutamic acid at position 94 of the T cell clone TCRα chains is critical to peptide recognition, two mutants of the HA 1.7 TCRα chain were constructed in which the glutamic acid at position 94 was mutated to alanine (HAαE94A) and lysine (HAαE94K), respectively. Expression of the HA1.7 TCRβ paired with the mutated HA1.7 TCRα chains was studied in the RBL system. The extracellular domains of the HA1.7 TCRβ chain, and wild-type or mutant TCRα chains, were fused to the transmembrane and cytoplasmic domains of the CD3ζ chain. TCRαβ pairs were co-transfected with a Neo-containing vector into RBL cells. Stable transfectat lines were selected with G418, and then

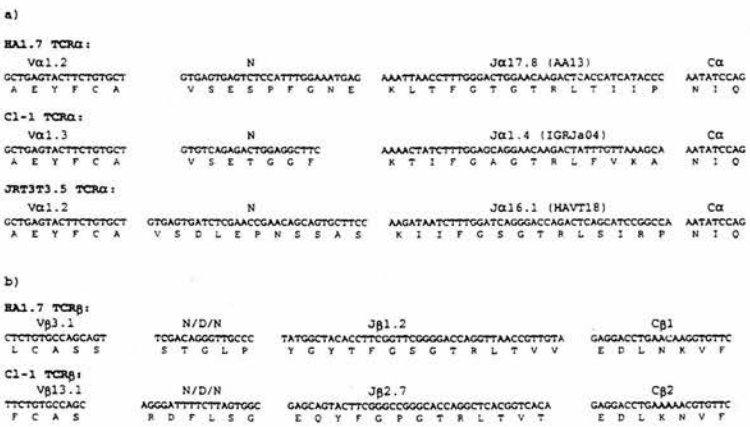
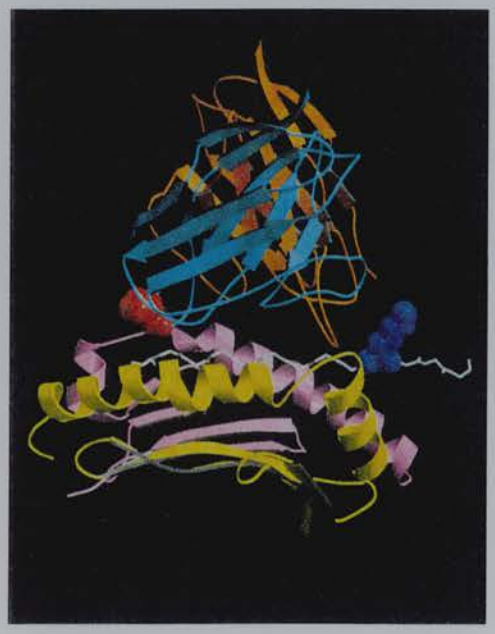


Figure 2. TCR sequences spanning the VJα and VDJβ junctions of T cell clones HA 1.7 and Cl-1, and of the T cell line JRT3T3.5 (Jurkat derivative). Nucleotide and predicted protein sequences of a) TCRα and b) TCRβ junctional regions are shown. Spaces have been inserted to allow alignment of conserved sequences.

a) b)



c)

Figure 3. Molecular models showing the predicted interaction between TCR HA1.7 and the HA 307–319/DR1 complex [3]. The Vα and Vβ chains are shown in blue and orange, respectively and the MHC α and β chains in pink and yellow, respectively. The peptide is shown in white and is orientated N terminus (right) to C terminus (left) in a) and b), and in the reverse orientation in c). a) side view of the TCR-MHC complex showing the proximity of residue TCRVα 94E (space-filled red) to peptide residue 316K; b) end view of the TCR-MHC complex obtained by rotation of view a) by 90°. The CDR3 loops of the Vα and Vβ chains can be seen to be located above the peptide, while the CDR1 and CDR2 regions are positioned over the MHC helices. c) view of the TCR-MHC complex in which the peptide-MHC complex has been rotated through 180° with respect to the TCR. Here, peptide residue 316K is seen to be outside the interaction interface. Figures were generated using MOLSCRIPT [39].

sorted by expression of the TCRβ chain. All three RBL transfectant lines, expressing wild-type HA1.7 TCR, mutant HA TCRαE94A or HA TCRαE94K, were sorted for identical levels of TCRβ expression, which was stable over many months *in vitro*. These lines also stained with the same intensity with the TCRα-specific antibody αF1. This antibody does not stain live T cells, but detects the TCRα chain in Western analysis. The staining by αF1 of RBL transfectants, which express TCR in the absence of CD3, suggests that the epitope recognized by αF1 is usually masked on T cells, perhaps by the CD3 complex.

Since the TCR-negative Jurkat line JRT3T3.5 is known to have a functional mRNA for the Jurkat TCRα chain, transfection of a TCRβ chain restores CD3 and TCR expression to these cells [21]. We have therefore taken advantage of the natural mutant of the Jurkat TCRα chain in which the residue at position 94 is another charged amino acid, aspartic acid 94D, similar to the E of the HA1.7 and Cl-1 TCRα chains. The hybrid HA1.7 TCRβ-Jurkat TCRα receptor was studied by transfection of the HA1.7 TCRβ into JRT3T3.5. Transfectants were selected in hygromycin, clones expressing CD3 and TCRβ were selected by flow cytometry, and compared to the HA1.7 wild-type transfectant CH7C17 by functional assays.

3.5 Recognition of the HA 307–319 peptide by wild-type and mutant TCR

RBL transfectants expressing the HA1.7 wild-type and mutant TCR were tested for their ability to respond to the HA 307–319 peptide in the context of DR1, and to other stimuli. All three lines responded equally well to the physiological stimulus of specific IgE and antigen (mediated by the FcεRI IgE receptor) or the nonspecific stimulus of phorbol ester plus ionomycin (data not shown). All transfectants also showed equivalent responses to TCR stimulation by the JOVI-3 antibody, showing that TCR triggering was functional in all three lines. The wild-type HA 1.7 TCR-positive cells responded specifically to HA 307–319 on DR1$^+$ APC, in a dose-dependent manner (Fig. 4), with no response to the irrelevant DR1-binding peptide MAT 19–31. The response to maximal peptide concentrations was equal to that obtained by triggering the TCR using the specific anti-TCR antibodies JOVI-1 or JOVI-3. However, TCR with mutant TCRα chains (HA TCRαE94A or HA TCRαE94K) showed no responses to the HA peptide, even at maximal doses, but were still able to respond well to JOVI-3 antibody (Fig. 5). None of the transfectants responded to an irrelevant isotype-matched control antibody (data not shown), or to APC or medium alone (Fig. 5).

The clone CH7C17, expressing the wild-type HA1.7 TCR, responds to HA peptide over the same dose range and with the same specificity as the original T cell clone (LRW, unpublished data, and [22]). We therefore tested transfectants of this cell line expressing the HA1.7 TCRβ chain paired with the Jurkat TCRα in the same way. Surprisingly, out of 10 clones selected, none responded to wild-type HA peptide in the context of DR1, although all could be stimulated by ligation of the TCR using low concentrations of anti-TCR Vβ3 (JOVI-3), TCR Cβ (JOVI-1) or CD3 (UCHT1) antibodies (Fig. 6).

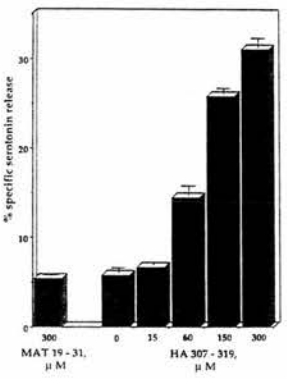


Figure 4. Stimulation of the wild-type HA1.7 TCRαβ pair expressed on RBL-2H3 cells. EBV-transformed B cells were loaded with HA 307–319 or MAT 19–31 at the given concentrations and used to present to [^{3}H]5HT-labeled HA1.7-TCR-transfected RBL-2H3 cells for 1 h. Results are expressed as % specific release = (cpm released/total lysate cpm + cpm released) × 100 ± SEM, of triplicate wells. Maximum cpm in lysate = 10165 ± 169.

3.6 Use of altered peptides to stimulate HA1.7 wild-type and mutant TCR

HA307–319 analog peptides with one altered residue at position 316 were tested in stimulation assays using RBL and Jurkat transfectants expressing HA1.7 wild-type or mutant TCR. The wild-type HA1.7 TCR-CD3 chimera expressed in the RBL system showed the same fine specificity for HA 307–319 altered peptides as the original clone. Notably, a weak response was obtained to the altered peptide 316C by wild-type TCR. However, other substitutions at position 316 created non-stimulatory peptides (Fig. 7).

The mutant TCR complexes were also tested for responses to altered peptides at this position. In particular, we reasoned that if the 316K directly contacts the oppositely charged 94E in the TCRα junctional region, then we might

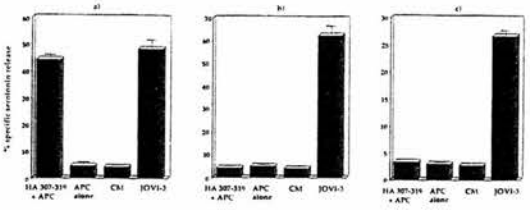


Figure 5. Loss of HA 307–319 recognition in TCR mutants bearing one altered residue in the junctional region of TCRα chain. The specific responses to maximal HA peptide (peptide loading concentration 300 μM) plus APC, APC alone, culture medium (CM) alone and anti-TCRβ antibody JOVI-3 (5 μg/ml) are shown for a) wild type HA1.7 TCR-transfectant, b) HAαE94A-TCR transfectant, and c) HAαE94K-TCR transfectant. Results are expressed as % specific release = (cpm released/total lysate cpm + cpm released) × 100 ± SEM, of triplicate wells. The cpm in total lysates were as follows: for a), 30756 ± 3621; b) 31275 ± 3043; c) 33201 ± 2276.

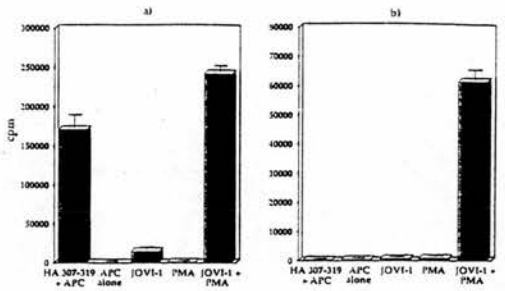


Figure 6. The Jurkat TCRα chain cannot replace the HA 1.7 TCRα chain. T cell responses in Jurkat derivatives were measured by lymphokine bioassay on CTLL cells. The responses of a) clone CH7C17 (HA1.7 TCRαβ transfectant), and b) clone H11 (Jurkat TCRα. HA1.7 TCRβ) are shown to: HA307–319 peptide on DR1 positive APC, APC alone, anti-TCR antibody JOVI-1 (1 μg/ml), PMA (0.5 ng/ml), and JOVI-1 plus PMA. Results are expressed as mean cpm ± SEM for triplicate wells.

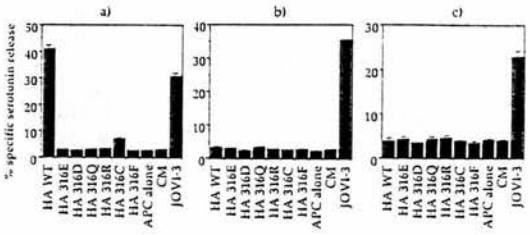


Figure 7. Mutant HA1.7 TCR bearing one altered residue in TCRα are not rescued by analog peptides with substitutions at position 316. The responses of RBL-transfected lines to HA 307–319 (HA WT) and 316-substituted peptides (HA 316E, etc.), on DR1 positive APC, as well as to APC alone, culture medium alone (CM), and the anti-TCR antibody JOVI-3 (5 μg/ml), are shown. a) wild type HA1.7 TCR transfectants, b) TCRα E94A mutant, c) TCRα E94K mutant. Results are expressed as % specific release = (cpm released/total lysate cpm + cpm released) × 100 ± SEM, of triplicate wells. The cpm in total lysates were as follows: for a), 34701 ± 3701; b) 28415 ± 4778; c) 30572 ± 2195.

be able to rescue peptide recognition by the E94K TCRα mutant with a complementary mutation in the peptide. However, none of the altered peptides tested were able to rescue the response of the altered TCR to the HA peptide. Specifically, no response was obtained from the HAα E94K mutant TCR to the peptide analogs 316E or 316D (Fig. 7).

4 Discussion

In this study, we compared the response of two human T cell clones, HA1.7 and Cl-1, both specific for the influenza haemagglutinin peptide 307–319, on two different presenting MHC molecules. We found that HA 1.7 is cross-reactive on DR4, while Cl-1 reacts only poorly to the HA/DR4 complex. We also showed that these two clones have TCR which use different TCRβ chains with no conservation of CDR3. In contrast, the TCRα chains of the two clones are closely realted both in terms of the gene segments used and in the presence of a highly conserved motif in the CDR3.

The two TCRα chains are Vα1.2 (TCRAV1S2A2) for HA1.7 and Vα1.3 (TCRAV1S3) for Cl-1, respectively, but the sequence of the Vα1.3 segment from Cl-1 has two amino acid changes just adjacent to the CDR2 region, which are altered such that they are identical to the equivalent residues in the Vα1.2. The conserved motif across the junctional region V-S-E-S/T- P/G contains a charged residue, glutamic acid. The highly related TCRα chain (Vα1.2) of the Jurkat derivatives has the sequence V-S-D-L-E over these five residues, with the related aspartic acid in position 94. However, the hybrid TCR of the Jurkat TCRα paired with the HA1.7 TCRβ chain cannot recognize the HA 307–319 peptide in the context of DR1. At the structural level, we cannot be sure of the conformational differences between the Jurkat and HA1.7 or Cl-1 TCRα CDR3 regions, since no structures are available. However, the substitution of D for E and the presence of the hydrophobic L immediately following the D would confer a different packing preference for this part of the CDR. In additon, this motif in the Jurkat sequence has an additional E in the fifth position compared with either P or G in the HA1.7 and Cl-1 sequences, respectively. The combination of these apparently small differences could have dramatic affects on the local CDR conformation and would be sufficient to explain the loss of specificity. Taken together, these data suggest that the TCRα chains of the two T cell clones may confer precise peptide specificity, but that even a small change in this region, such as that created by the substitution of the Jurkat TCRα chain, leads to a loss of recognition.

Several previous studies have implicated complementary charge interactions as important in the interactions between TCR and specific peptide, both in murine [15], and human T cells [12, 36]. For example, in the analysis of the TCR 5C.C7, specific for pigeon cytochrome c in the context of I-E^k, a glutamic acid residue at position 93 of the TCRα chain interacts with the residue 99K in the peptide (MCC 88–103). In mice transgenic for the TCRβ chain of 5C. C7, immunization with the variant peptide MCC 99E produced hybridomas expressing related TCRα chains, in which position 93 was replaced by a lysine [15].

In the analysis of TCR specific for HA 307–319, previous studies [17, 19, 20] and our own work (LRW and JRL, unpublished) have characterized residues 308K, 311K, 313N, 316K, and 319T as important for TCR contacts. With the availability of the crystal structure of the HA 307–319/DR1 complex, we have been able to model the TCR described in this study, and have predicted that the TCRα residue 94E lies close to 316K of the peptide. To test this, we have constructed mutants at this position and expressed these and the wild-type HA 1.7 TCR in the RBL-2H3 system, using TCR-CD3ζ chimeric constructs. In this cell line, stable and high cell-surface expression of transfected constructs can be achieved. The CD3ζ chain allows heterodimerization of the two chains, and mediates a signal which leads to basophil granule exocytosis, due to its homology with the physiological signaling molecule in the basophil, the FcεRI [37].

In this study, peptide-specific recognition by TCR has been reconstituted in the RBL-2H3 cell line by transfection of the wild-type HA 1.7 TCR. Two mutant TCRα chains, with residue 94 altered to A and K respectively, have also been tested. Both TCR carrying mutant α chains have good responses to TCR ligation by anti-TCRβ antibody stimulation. As expected from the prediction, the TCRα E94A and E94K mutants show an almost total loss of response to HA 307–319, indicating the critical nature of the 94E residue in binding the peptide/MHC ligand.

In an attempt to obtain further evidence for the orientation of the HA1.7 TCR on its ligand, we have tested the responses of the wild-type and mutant TCR to altered peptides in which 316K has been substituted with other residues. In parallel with the original HA 1.7 clone, the wild-type TCR shows a weak response to the 316C analog, but no response to 316R. The HA1.7 T cell clone responds to 316R, albeit poorly; however, this requires very high concentrations of peptide, and it is likely that the affinity of the TCR for this peptide in the context of DR1 is too low to produce a signal in the RBL system.

The use of altered peptides to stimulate the mutant TCR could not rescue recognition. In particular, it was not possible to restore the electrostatic partner of the TCRα E94K mutant using the altered peptides 316E and 316D. This is in contrast to the 5C.C7 TCR transgenic mice, where reciprocal charge exchange restored responsiveness [15]. However, it is not always possible to introduce such reciprocal mutations without some disruption of the surrounding polypeptide chain, since the structural context in which a charged residue is placed can be a critical determinant of local conformation. In fact it has been suggested that ion-pair reversal in proteins is "unlikely to succeed" [38]. The difficulty of this type of charge engineering is shown by the fact that even a conservative change can have a profound biological effect, as shown by the greatly reduced response of the wild type HA1.7 TCR to the K316R peptide.

This study adds to the evidence that the CDR3 sequences of TCR chains, in this case the TCRα chain, play a major role in the specific recognition of antigenic peptide. Our model also predicts that the difference in DR cross-reactivity between the two clones could be explained by the differing TCRβ chains. The class II molecule DRB1*0404 differs from DRB1*0101 by only 10 residues. The majority of the polymorphic residues which differ between these two DR molecules lie in the base of the peptide binding groove, such as 11, 13, 28, 30, 31, and 37_β. In DR1, these residues do not form direct interactions with side chains of the peptide [3]. While the difference between the clones may be mediated by direct interaction between the TCRβ chains and the polymorphic residues of the DR molecule, it is also possible that the shape of the HA peptide differs when presented in the context of DR4 instead of DR1, and that TCRβ – peptide contacts are different between the two ternary complexes. This has not formally been tested in the present study, and further experiments are in progress to address this issue.

We thank Dr. R. O'Hehir for advice and help with the T cell clones. We thank Dr. H. Grey, Prof. A. Weiss, Dr. C. Hewitt, Dr. J. Viney, Dr. J. Bodmer, Prof. P. Beverley, and Dr. T. Ottenhoff for gifts of reagents. We thank Oxford Molecular Ltd. for computer hardware. This work was supported by the Imperial Cancer Research Fund (LRW, MJO), the Wellcome Trust (JRL), and the SERC (SJMJ). The work was funded in part by an EC biotechnology grant (no BIO2CT920164) to MJO.

Received March 13, 1995; accepted April 11, 1995.

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Binary and ternary complexes between T-cell receptor, class II MHC and superantigen *in vitro*

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SUPERANTIGENS are proteins that in association with class II major histocompatibility complex (MHC)-bearing cells can stimulate virtually all T cells that express particular classes of the variable β-domains of the T-cell receptor (TCR)[1]. This mechanism of T-cell activation circumvents the usual requirement for peptide-specific MHC recognition. *Staphylococcus aureus* enterotoxin B (SEB) is a bacterial superantigen that causes food poisoning and shock[2–5]. We have characterized the tertiary complex of SEB, a soluble T-cell receptor, and a soluble class II MHC molecule DR1, and the three binary complexes TCR–SEB, SEB–DR1, and the peptide-specific complex DR1–TCR. We report here that in each case the specificity of the interaction among the soluble molecules is the same as observed in biological assays. Native gel electrophoresis and plasmon resonance affinity measurements indicate that SEB–TCR complex can form in the absence of class II MHC and that SEB–TCR interaction increases the binding of DR1. The observation that a superantigen can form complexes with TCR in both the absence and presence of class II MHC may provide a mechanism for its ability to induce anergy in some circumstances and activation in others[6,7] (reviewed in ref. 8).

To investigate the interactions of TCR, class II MHC and SEB, we used a soluble human TCR $\alpha\beta$ heterodimer (TCR HA1.7) which has specificity for influenza haemagglutinin HA peptide (306–318) bound to the human class II MHC molecule HLA-DR1[6]. The variable (V) gene segment of the β-chain of TCR (HA1.7) is Vβ3.1, which has specificity for SEB[9]. A native gel-shift assay (Fig. 1) was used to examine formation of complexes between TCR, DR1 and SEB under non-denaturing conditions. TCR (lane 1), SEB (lane 4), and DR1-HA (HA peptide 306–318 bound to HLA-DR1[10]) (lane 6), migrate through a native polyacrylamide gel with distinct mobilities. Co-incubation of TCR, DR1-HA and SEB resulted in a new band (lane 2,***), indicating formation of a complex. The presence of TCR, DR1 and SEB in this band was confirmed by immunoblot analysis using antibodies specific for each protein (Fig. 1*b*). These data confirm the existence of the predicted ternary complex[5].

No complexes are observed under these conditions between DR1 and SEB in the absence of TCR (Fig. 1*a*, lane 3) or between DR1–HA and TCR in the absence of SEB (Fig. 1*a*, lane 5). This could result from weaker affinities and/or fast dissociation rates of these two binary complexes, which are beyond the sensitivity of detection in native gels. Affinity of the TCR/MHC class II interactions has been reported to be low (dissociation constant $K_D \sim 10^{-4}$ to 10^{-5} M)[11–13].

The binary complex between SEB and TCR (in the absence of DR1) is detected as a band on native gel electrophoresis (Fig. 2*a*, lane 6,**) with mobility distinct from that of SEB (lane 7) or TCR (lane 8).

The specificity of the interactions among soluble TCR, superantigen and class II MHC molecules was also studied with native gel electrophoresis. The ternary complex TCR–SEB–DR1 was observed with four different peptides bound to DR1 (Fig. 2*a*; compare lanes 1–4 to lanes 10–13). This indicates that *in vitro*, as *in vivo*, superantigen circumvents the usual requirement for peptide-specific MHC recognition by TCR. The slight variation in mobility of the ternary complex bands (lanes 1–4,***) reflects differences in the mobilities of HLA-DR1 bound to different peptides (lanes 10–13). This observation, as well as the disappearance of the DR1 and TCR bands in lanes 1–4 (Fig. 2*a*), provides additional evidence for the presence of all three protein molecules in the ternary complex band.

The specificity of this TCR (HA1.7, Vβ3.1) for the superantigen SEB[14] but not SEA (*S. aureus* toxin A) or TSST-1 (toxic-shock-syndrome toxin 1)[15] was observed with native gel electrophoresis. SEB formed a complex with TCR in both the absence (Fig. 2*a*, lane 6; Fig. 2*b*, lane 3) and presence (Fig. 2*a*, lane 4,***) and Fig. 2*b*, lane 4) of DR1, whereas TSST-1 (Fig. 2*b*, lanes 5, 6) and SEA (not shown) show no complex formation with this TCR. Immunoblot analysis with a TCR antibody (Fig. 2*c*) confirmed that this TCR did not form a complex with TSST-1 in the absence (lane 2) or presence (lane 3) of DR1.

We have also observed the ternary and binary complexes using a BIAcore instrument[16,17] that can detect the binding of one or more soluble proteins to a protein immobilized on a dextran-coated chip as a difference in refractive index[18]. Figure 3 shows the binding of proteins to immobilized TCR. SEA (1 μM) shows no significant binding, whereas SEB (1 μM) clearly forms a complex with TCR (Fig. 3). DR1-HA at high concentration (25 μM) shows a small binding signal, whereas DR1-HA (1 μM) pre-mixed with SEB (1 μM) binds to the TCR surface with a large signal (much greater than additive), indicating formation of the ternary complex (Fig. 3*a*). In control experiments, neither SEB nor DR1-HA bound significantly to the dextran matrix alone; and other proteins for which the TCR is not specific, such as DR1-I24 or HLA-A2 (class I MHC), did not bind to the immobilized TCR (Fig. 3*b*, and data not shown). Although high DR1-HA concentration (25 μM; Fig. 3) was required to observe binding to immobilized TCR, the binding was peptide-specific. DR1 complexed with another peptide (I24) showed no binding at 25 μM (Fig. 3*b*). On the basis of experiments using DR1-HA concentrations up to 50 μM, we estimate that the K_D of the DR1-HA, TCR (HA1.7) interaction is greater than 25 μM *in vitro*.

With SEB immobilized on a chip, both the binary complexes DR1–SEB and TCR–SEB were readily detected (Fig. 4*A*, *B*). In control experiments, neither DR1 (Fig. 4*Aa*, curve 2) nor TCR (not shown) bound to the free dextran surface; and HLA-A2 (class I MHC), which does not have specificity for SEB, did not bind to the immobilized SEB (not shown). In Fig. 4*A* a binary complex between DR1 and SEB is observed, which was not detectable by native gel electrophoresis (see above). Figure 4*B* provides further evidence that SEB can form a stable binary complex with TCR in the absence of MHC class II.

Equilibrium dissociation constants and kinetic on- and off-rates were measured for the novel TCR–SEB complex. Kinetic constants were calculated from the ascending rate of the BIAcore signal during binding and the descending rate during wash-off (Fig. 4, legend). For TCR binding to immobilized SEB, an on-rate constant ($k_{on} = 1.30 \pm 0.12 \times 10^4\ M^{-1}\ s^{-1}$) and off-rate constant ($k_{off} = 1.06 \pm 0.05 \times 10^{-2}\ s^{-1}$) were determined from triplicate experiments (Fig. 4*B*). From their ratio, the equilibrium dissociation constant, $K_D = 0.82$ μM, is estimated for the TCR–

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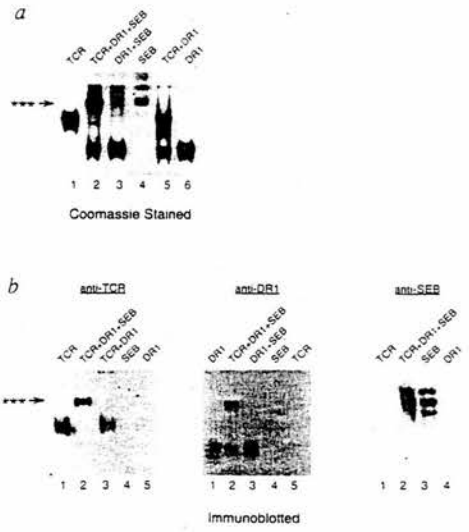

FIG. 1 Native gel shift assay. *a*, Co-incubation of DR1-HA (HLA-DR1 bound to HA 306–318 peptide), SEB and TCR (lane 2), results in the appearance of a distinct band (▪▪▪), not observed with TCR (lane 1), SEB (lane 4) or DR1-HA (lane 6) alone, indicating TCR–SEB–DR1 complex formation. Co-incubation of DR1-HA and SEB (lane 3) or of DR1-HA with TCR (lane 5) did not result in any distinct mobility shift. *b*, Anti-TCR blot (αF1 mAb; T Cell Sciences) shows that TCR is shifted by incubation with DR1-HA and SEB; anti-DR1 blot (L243 mAb American Type Culture Collection II HB55) shows that DR1-HA is shifted by incubation with TCR and SEB; anti-SEB blot (polyclonal serum; Toxin Technology) shows that SEB is shifted by incubation with TCR and DR1-HA. Thus all three proteins are present in the band (▪▪▪).
METHODS. HLA-DR1 (DRB1*0101), was produced in insect cells as a soluble protein, purified and complexed *in vitro* with peptides as published[10]. A soluble human $\alpha\beta$ T-cell receptor (HA1.7, Vβ3.1 Vα1.2) was expressed and isolated as previously described for a mouse TCR[21] (I.E. and T.O., unpublished results). SEB was purchased from Sigma. The TCR, peptide-complexed DR1 and SEB used in these experiments appear homogeneous by gel-filtration HPLC and by SDS–PAGE. The multiple SEB bands in native-PAGE may result from charge heterogeneity. Non-denaturing native-PAGE was done by omitting SDS in a PAGE system[22]. Proteins (15 μM TCR, 20 μM DR1, 35 μM SEB) were incubated alone, or in combination, in PBS, with 1 mM phenylmethylsulphonyl fluoride, 10 μg ml^{-1} leupeptin, pH 7.0, at 37 °C for 18 h before non-reducing native polyacrylamide (10%) gel electrophoresis. No reducing agents were added and samples were not heated. The resolving gel buffer was 0.375 M Tris, pH 8.8. In native-PAGE the direction of migration of the proteins will depend on the buffer pH relative to the isoelectric point (pI) of the protein[23]. The gels were run at 15 V cm^{-1}. Protein bands were visualized with *a*, Coomassie brilliant blue R250, or *b*, were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore), probed with specific antibodies, and the immune complexes visualized by alkaline phosphatase[24].

SEB complex. A similar value (K_D = 0.68 μM) was obtained from a Scatchard analysis (not shown) of equilibrium binding at various TCR concentrations (Fig. 4*Ba*). The equilibrium dissociation constant for the DR1-SEB complex was also measured. Scatchard analysis (Fig. 4*Ac*) of the data in Fig. 4*Ab* gave a K_D = 1.7 μM, a value similar to that measured for SEB binding to DR1 on cell surfaces[19].

The kinetics of DR1-SEB association and dissociation were too fast to be measured accurately in the experiments shown in Fig. 4*A*. The fast dissociation kinetics may be the reason that the DR1-SEB complex was not detectable in the native gel-shift assay (see above).

Our native gel electrophoresis results indicate that DR1 with peptide binds better to the SEB-TCR complex than to either molecule separately. TCR-SEB-DR1 complexes could be observed for four different DR1-peptide complexes under condi-

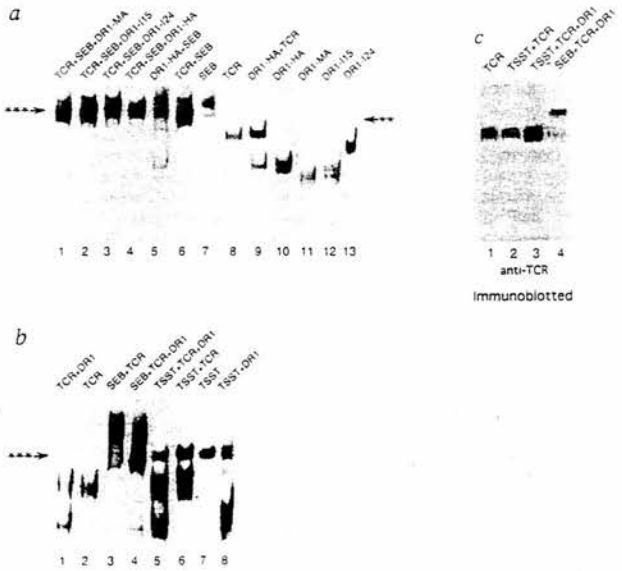

FIG. 2 Specificity of TCR–SEB and TCR–SEB–DR1 complex formation. *a*, In the presence of SEB, TCR migrates with a mobility (lane 6, ▪▪) intermediate between SEB (lane 7) or TCR (lane 8) alone. Formation of the TCR–SEB–DR1 complex (lanes 1–4, ▪▪▪) was independent of the peptide bound to DR1 (peptide sequences listed below), and was accompanied by a band shift relative to peptide-bound DR1 (lanes 10–13) and TCR alone (lane 8). *b*, The interaction of TCR with SEB was compared to the interaction of TCR with TSST-1. No complexes were detected when TCR was incubated with TSST-1 (lanes 5 and 6) or SEA (data not shown), but TCR–SEB (lane 3) and TCR–SEB–DR1 (lane 4, ▪▪▪) complexes were observed. Neither TSST-1 nor SEA are reported to interact with Vβ3.1 (ref. 1). *c*, Anti-TCR immunoblot of native gel with αF1 mAb specifically confirms that TCR is not shifted by incubation with TSST-1 (lane 2) or TSST-1 + DR1 (lane 3), but is shifted by incubation with SEB and DR1 (lane 4).
METHODS. Proteins (10 μM each) were incubated, subjected to native-PAGE (8%), and visualized as in Fig. 1. *a* and *b*, Coomassie stained. *c*, Anti-TCR immunoblot. Peptides: HA, influenza haemagglutinin (306–318) PKYVKQNTLKLAT; I24, class II-associated invariant chain (96–119), LPKPPKPVSKMRMATPLLMQALPM; I15, class II-associated invariant chain (105–119), KMRMATPLLMQALPM; MA, influenza matrix (17–29), SGPLKAEFAQRLE.

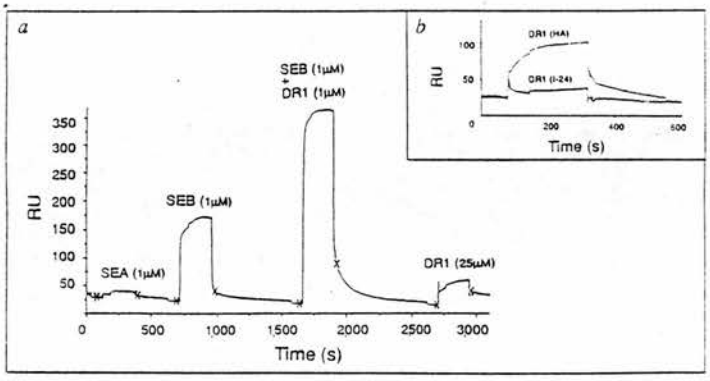

FIG. 3 Interaction of SEB, SEA, DR1 and premixed (DR1 + SEB) to immobilized TCR. Protein–protein interactions detected by surface plasmon resonance (BIAcore instrument, Pharmacia), where increase in RU (resonance units) indicates binding of injected protein to protein immobilized on surface. *a*, SEB (1 μM) but not SEA (1 μM) binds to immobilized TCR. Although DR1-HA binds only weakly even at concentrations as high as 25 μM, premixing of SEB plus DR1-HA at equimolar (1 μM) concentrations before injection resulted in a more than additive increase in binding signal. *b*, Overlaid sensorgrams comparing binding of immobilized TCR to DR1 bound to the correct antigenic peptide (DR1-HA, 25 μM) versus DR1 bound to the control peptide (DR1-I24, 25 μM).

METHODS. TCR was coupled to the dextran matrix by standard amine chemistry[25]. A flow of HBS (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.5% surfactant P20 pH 7.4) was maintained over the sensor surface at 5 μl min^{-1}. Samples were injected at 5 μl min^{-1} for 5 min each. Between injections, the surface was regenerated with HBS. The immobilization level was 3,000–4,000 RU. 'X', Resonance signal before the injection, at the height of the response during the injection and towards the end of the wash. Although binding of DR1 and SEB to the immobilized TCR is detectable, baseline drift made kinetic and affinity measurements impractical.

tions where TCR–SEB complex formed, but DR1–SEB complexes were not stable (Figs 1 and 2). This suggests that either soluble $\alpha\beta$TCR alters the conformation of SEB to a form which has higher affinity for DR1, or that $\alpha\beta$TCR directly contacts parts of the DR1 molecule in the TCR–SEB–DR1 complex. Such a direct contact has previously been suggested based on variations in T-cell responses with MHC class II polymorphism, with the conformational state of MHC class II, and with the type of TCRα chain (for review see ref. 20); this contact could occur on a DR1 surface adjacent to the bound peptide.

Two observations reported here indicate that TCR–SEB–DR1 complexes can be formed from soluble molecules. First, native gel electrophoresis of mixtures of the three molecules reveals a band with mobility distinct from the three individual constituents, or their binary complexes, and that band has been shown by immunoblotting to contain all three molecules (Figs 1 and

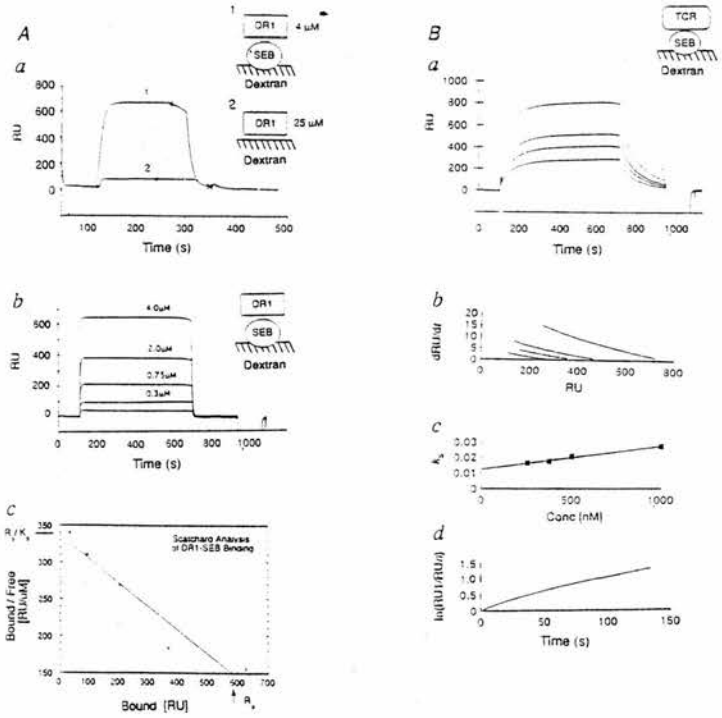

FIG. 4 Measurement of binding parameters for DR1–SEB (*A*) and TCR–SEB (*B*) complexes. *Aa*, DR1 binding to SEB surface (trace 1) versus control surface (trace 2); *b*, Concentration-dependent binding of DR1 to SEB surface; *c*, Scatchard plot intercepts give R_0 (maximum DR1-HA bound) and R_0/K_D[26], yielding a K_D of 1.7 μM. *Ba*, Bottom to top traces, TCR ~ 0.25 μM, 0.35 μM, 0.5 μM, 1.0 μM, binding to SEB. *b*, Binding rates dRU/dt at these TCR concentrations versus relative response in resonance units, RU. *c*, Association rates (k_s) versus TCR concentration (k_s = slopes from *b*). An apparent association rate constant ($k_{on} = 1.30 \pm 0.12 \times 10^4\ M^{-1}\ s^{-1}$; $n = 3$) was calculated for TCR binding to SEB. *d*, Dissociation of bound TCR (1 μM trace) from immobilized SEB. Assuming a single exponential decay, $k_{off} = 1.06 \pm 0.05 \times 10^{-2}\ s^{-1}$ ($n = 3$). The ratio of off- and on-rate constants gives an apparent equilibrium dissociation constant $K_D = 0.8$ μM. The data shown here are from a typical experiment. The values for kinetic constants are mean ± s.d. from n independent experiments. Methods as in Fig. 3.

2). Second, plasmon resonance measurements of SEB and DR1 binding to an immobilized TCR indicate that much more DR1 binds to the immobilized TCR in the presence of SEB than binds under the same conditions in the absence of SEB (Fig. 3). We also confirm the weak affinity for MHC class II with peptide to TCR[11-13], demonstrating here that both soluble proteins interact in a peptide-specific manner as required for MHC restriction. Finally, we have observed direct binding of SEB to $\alpha\beta$TCR in the absence of a class II MHC molecule ($K_D = 0.82\ \mu M$) which exhibits kinetics ($k_{on} = 1.30 \times 10^4\ M^{-1}\ s^{-1}$; $k_{off} = 1.06 \times 10^{-2}\ s^{-1}$) that suggest a half-life of the complex of 90 s. Although several studies report that activation of T cells by superantigen requires the presence of MHC class II molecules, under certain circumstances (crosslinking of toxin and/or presence of co-stimulant) superantigen can activate T cells in the absence of class II molecules (reviewed in refs 1, 8, 15). In the absence of co-stimulants or crosslinking, SEB alone appears to cause anergy[6]. The finding that superantigen complexes with TCR in both the presence and the absence of class II MHC *in vitro* may correlate with these different activities *in vivo*. □

Received 24 December 1993; accepted 29 March 1994.

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ACKNOWLEDGEMENTS. We thank D. Garboczi and T. Jardetzky for discussion; J. Strominger and colleagues for collaborative studies of class II MHC molecules; D. Garboczi for reading the manuscript; M. Frayser for technical assistance; and R. Reed of Pharmacia Biosensor for his assistance with the Biacore measurements. T.O. is sponsored by the Netherlands Organization for Scientific Research NWO. The research of A.S., L.S. and D.C.W. is supported by the Howard Hughes Medical Institute and by the NIH. D.C.W. is an investigator of the Howard Hughes Medical Institute.

2.2 MODULATION OF ACCESSORY/CO-RECEPTOR MOLECULES

Faith A et al: *Clin Exp Allergy* 1995, **25**:1163-1170.

Lamb JR et al: *Clin Exp Allergy* 1995, **25**:839-847.

Clinical and Experimental Allergy, 1995, Volume 25, pages 1163–1170

Differential dependence of TH-0, TH-1 and TH-2 CD4+ T cells on co-stimulatory activity provided by the accessory molecule LFA-1

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Summary

Background The adhesion molecule LFA-1 contributes to the activation response of peripheral blood human CD4+ T cells. Less is known of its contribution to stimulation of long-term CD4+ T cell lines and clones or of its potential to co-stimulate CD4+ T cells of different functional phenotype.

Objective This study was therefore performed to investigate co-stimulatory properties of the LFA-1 (CD11a/CD18) complex in the activation of human CD4+ T cell lines and clones of TH-0, TH-1 and TH-2 subsets.

Methods Co-stimulatory activity was measured by cross-linking antibodies to CD11a or CD18 with anti-CD3 antibodies to plastic and then measuring the proliferative response of CD4+ T cells to these antibodies.

Results A house duct mite allergen-specific CD4+ T cell line (TH-2) demonstrated much greater dependence on both CD11a and CD18 than a mycobacterial antigen-specific CD4+ T cell line (TH-1). Co-stimulatory activity through LFA-1 was also provided to a house dust mite-specific CD4+ T cell clone (DE-9; TH-2) but not to an influenza haemagglutinin-specific CD4+ T cell clone (HA1·7; TH-0). In contrast, soluble antibodies to CD18 inhibited proliferative responses of both DE-9 and HA1·7 to an immunogenic challenge of antigen and to stimulation by anti-CD3 antibodies. However, the allergen-specific T cells were more susceptible to inhibition. Signal transduction was also observed from the T-cell receptor to LFA-1. Ligation of the T-cell receptor modulated the phenotypic expression of LFA-1 and ICAM-1 on both HA1·7 and DE-9. Phenotypic modulation was observed as a result of both activation and the induction of non-responsiveness.

Conclusion These experiments indicate that CD4+ T cells of TH-2 functional phenotype may have a greater requirement for the co-stimulatory activity of LFA-1 than CD4+ T cells of TH-0 or TH-1 phenotypes.

Keywords: CD4+ T cells, TH-0, TH-1, TH-2, functional phenotype, LFA-1, ICAM-1

Clinical and Experimental Allergy, Vol. 25, pp. 1163–1170. Submitted 20 May 1994; accepted 11 October 1994.

Introduction

Antigenic peptides are presented to CD4+ T cells by MHC class II molecules expressed on the membrane of accessory cells [1]. Accessory cells also bear several ligands which interact with complementary receptors on T cells. These molecular associations provide the activation-dependent adhesion contributing to enhanced responsiveness characteristic of CD4+ T cells. Several major families of adhesion molecules have been described [2], the best characterized of which are the integrins and the immunoglobulin superfamily. The

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integrins can be divided into several families of related molecules, possessing a unique α and common β chain. The $\beta 2$ integrin LFA-1 (CD11a/CD18) [3] is present on T cells and contributes to activation and proliferation. The counter receptor for LFA-1 is a member of the immunoglobulin superfamily ICAM-1 (CD54) [4], and is expressed on a variety of tissues including peripheral blood leucocytes, vascular endothelium and epithelial cells. Recently the counter receptors ICAM-2 and ICAM-3 have been described [5,6]. ICAM-2 is the predominant LFA-1 binding ligand on resting endothelium while ICAM-3 although absent from endothelial cells is strongly expressed on all leucocytes.

There is evidence to suggest that the role of the adhesion molecules may not be limited to forming a molecular bridge between accessory cells and T cells. ICAM-1 has recently been shown to provide co-stimulation in response to anti-CD3 antibodies by prolonging inositol phospholipid hydrolysis [7].

Murine CD4+ T cells have been divided into two mutually exclusive subsets, depending on their lymphokine profiles [8]. There is now strong evidence that human CD4+ T cells, derived from atopic individuals and responding to house dust mite (HDM) allergens, belong to the TH-2 functional phenotype [9], secreting cytokines such as IL-4, IL-5 and IL-10. However, most human CD4+ T cell clones, derived from normal individuals and responding to recall antigens, belong to the TH-0 phenotype producing high levels of IFNγ as well as TH-2 cytokines [10]. It is not known whether the role of accessory molecules, in activation of human CD4+ T cells belonging to different functional phenotype, differs. We have, therefore, compared the function of LFA-1 in the activation of CD4+ T cells of TH-0, TH-1 and TH-2 phenotype, respectively.

Materials and methods

Antibodies

Anti-CD3 antibodies, affinity purified by Protein-A Sepharose chromatography, was the kind gift of Dr H. Spits, Cancer Research Centre, Amsterdam, the Netherlands. Purified anti-CD11a (38; IgG2a) and purified anti-CD18 antibodies (60.3: IgG2a) were kindly provided by Dr N. Hogg, Imperial Cancer Research Fund, London, UK and Dr J. Ledbetter, Bristol-Myers Squibb, Seattle, USA, respectively.

Antigens

Staphylococcal enterotoxins (SE) B and E were purchased from Toxin Technology (Madison, WI, USA). The HA307-319 (HA307) C-terminal peptide of the influenza virus haemagglutinin antigen was prepared using standard solid-phase methods. Lyopholized extracts of the house dust mite *Dermatophagoides pteronyssinus* were kindly provided by SmithKline, Beecham (Brentford, UK). Recombinant *Der p* II was kindly provided by Dr W. R. Thomas, Princess Margaret Hospital, Perth, Western Australia. Mycobacterial soluble extract (MTSE) was kindly provided by Professor D. Young, Department of Microbiology, St Mary's Hospital Medical School, London, UK.

T cell lines

Peripheral blood mononuclear cells (PBMC; 2×10^6/mL) were stimulated with *Der p* II (10 μg/mL) or MTSE (5 μg/mL) for 10 days and viable cells recovered from Ficoll-Hypaque gradients and restimulated (1×10^6/mL) with antigen in the presence of autologous, irradiated PBMC (APC; 1×10^6/mL) and 5% Lymphocult-T (Biotest Serum Institute, Frankfurt, Germany). T cells were expanded by alternate weekly cycles of stimulation with antigen, APC and IL-2 or IL-2 alone. The T cell lines were used in assays after 3 weeks stimulation when CD4+ T cells represented >90% of each population. The MTSE-specific line secreted 18250 pg/mL of IFNγ and 20 pg/mL of IL-4 following restimulation after 2 weeks in culture and was therefore of TH-1 functional phenotype. The *Der p* II-specific line was established from an atopic donor.

T cell proliferation assays

The isolation and characterization of the cloned human T cells, HA1.7 and DE-9, reactive with HA307 and *Dermatophagoides pteronyssinus*, respectively, have been described elsewhere [11,12]. HA1·7 and DE-9 (1×10^5/mL) were cultured with HA307 (2 μg/mL) or *Dermatophagoides pteronyssinus* (10 μg/mL), respectively, with MHC class II matched, allogeneic, irradiated PBMC (5×10^5/mL) (APC), in the presence or absence of varying concentrations of antibodies to CD11a and CD18. For assays of co-stimulatory activity, varying concentrations of anti-CD3 antibodies and antibodies to CD11a or CD18 (10 μg/mL) were co-immobilized by coating round-bottomed 96-well microtitre plates, for 1 h at 37°C. Anti-CD3 antibodies were added first for 1 h to avoid competition with anti-LFA-1 antibodies for binding to the plastic. The plates were then washed twice with phosphate-buffered saline (PBS) and T cell clones or T cell lines (1×10^5/mL) added to wells. T cell clones (1×10^5/mL) were also stimulated with cross-linked anti-CD3 antibodies (10 μg/mL) in the presence or

absence of varying concentrations of soluble antibodies to anti-CD11a and CD18. For all assays, T cells were incubated for 66 h and proliferation measured by addition of ^{3}H-TdR during the final 16 h of culture. Proliferation is shown as the mean of triplicates ± standard error of the mean.

The induction of non-responsiveness was achieved by culturing HA1.7 (2×10^6/mL) with HA307 peptide (50 μg/mL) or SEB (0·5 μg/mL) and DE-9 (2×10^6/mL) with SEE (5 μg/mL) for 16 h. Cloned T cells were then washed and restimulated with an immunogenic challenge of their respctive antigens.

Fluorescence flow cytometry

T cells were stained directly with saturating concentrations of fluorescein-conjugated murine monoclonal antibodies, anti-Leu 4 (CD3) and anti-IL-2 receptor (CD25) antibodies (Becton Dickinson), using a mouse IgG1 fluorescein isothiocyanate (FITC)-conjugate control, or indirectly with anti-CD11a, anti-CD18, and anti-CD54 antibodies, and FITC-conjugated goat anti-mouse Ig (DAKO). Viable cells, identified by their ability to exclude propidium iodide, were analysed by flow cytometry using an Epics Prolife II (Coulter). The cell population was analysed by gating on the volume and light scatter characteristics.

Results

Effects of antibodies to LFA-1/ICAM-1 on antigenic responses of CD4+ T cell clones

Different functional populations of CD4+ T cells may have selective requirements for adhesion molecules. Therefore, we examined the role of these molecules in antigen presentation to T cell clones representative of the TH-0 and TH-2 functional phenotypes. The addition of soluble antibodies to CD11a and CD18 resulted in dose-dependent inhibition of the antigenic responses of both T cell clones (Fig. 1) but whereas the response of DE-9 was ablated at a concentration of 20 μg/mL of each antibody that of HA1.7 was reduced by only 50%.

Effects of antibodies to CD11a/CD18 on CD4+ T cell responses in the absence of accessory cells

Proliferation of polyclonal T cells, induced by cross-linked anti-CD3 antibodies, has been reported to be independent of the presence of accessory cells [13] and therefore, the contribution of adhesion molecules such as LFA-1 to CD4+ T cell activation can be directly assessed. Soluble antibodies to anti-CD18 gave

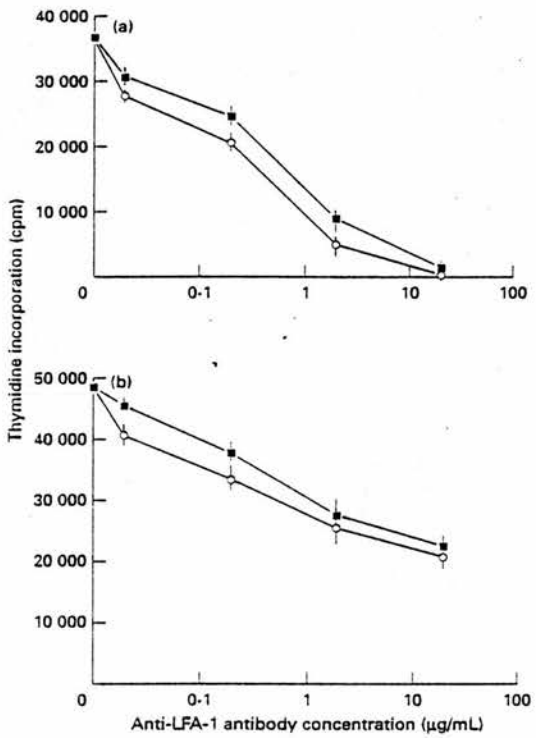

Fig. 1. Effect of antibodies to CD11a/CD18 on the response of the T cell clones, HA1.7 and DE-9, to antigenic stimulation. (a) DE-9 and (b) HA1.7 were stimulated with HA307 peptide and *D. Pter.*, respectively, in the presence of MHC class II matched, irradiated, allogeneic PBMC. Varying concentrations of antibodies to CD11a and CD18 (0·02–20 μg/mL) were added to the culture system. Cells were cultured for 66 h and tritiated thymidine incorporation measured during the final 16 h of culture. –■–. Anti-CD11a. –○–. Anti-CD18.

dose-dependent inhibition of the responses of both DE-9 and HA1.7 to cross-linked anti-CD3 antibodies (Fig. 2), but DE-9 was again much more susceptible to inhibition. Soluble antibodies to CD11a had no effect on the responses of either T cell clone.

Co-immobilization of antibodies to adhesion molecules and anti-CD3 antibodies have been shown to provide a co-stimulatory signal to polyclonal CD4+ T cells [14]. Antibodies to CD11a and CD18, cross-linked together with anti-CD3 antibodies, delivered a co-stimulatory signal, resulting in enhanced proliferation, to DE-9 at sub-optimal concentrations of anti-CD3 antibodies (Fig. 3a). In contrast, neither anti-CD11a or

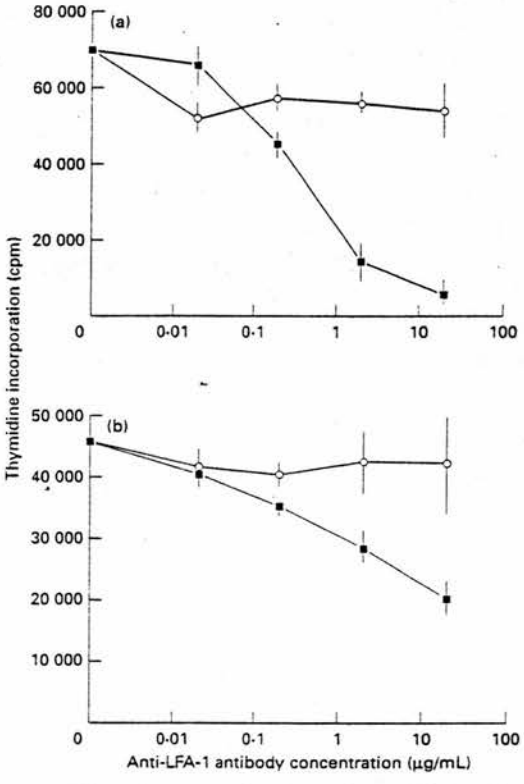

Fig. 2. Effect of soluble antibodies to CD11a/CD18 on the response of DE-9 (a) and HA1.7 (b) to anti-CD3 antibodies. Anti-CD3 antibodies (10 μg/mL) were immobilized on plastic wells. T cells (1×10^5/mL) and varying concentrations of antibodies to CD11a and CD18 (0·02–20 μg/mL) were added to each well, and proliferation measured as in Fig. 1. –■–. Anti-CD3 + anti-CD18. –○–. Anti-CD3 + anti-CD11a.

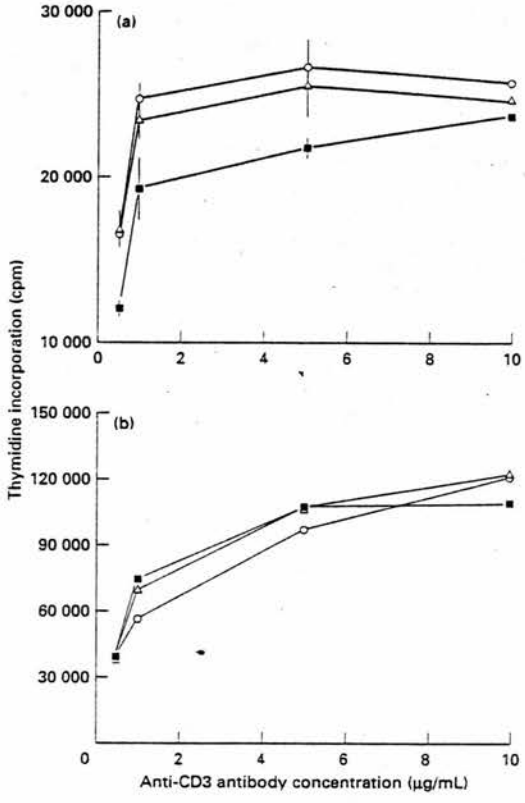

Fig. 3. Effect of cross-linked antibodies to CD11a/CD18 on the response of DE-9 (a) and HA1.7 (b) to anti-CD3 antibodies. Varying concentrations of anti-CD3 antibodies were co-immobilised on plastic wells with antibodies to CD11a or CD18 (10 μg/mL). T cells (1×10^5/mL) were added to each well and proliferation measured as in Fig. 1. –■–. Anti-CD3. –△–. Anti-CD3 + anti-CD18. –○–. Anti-CD3 + anti-CD11a.

CD18 antibodies had any effect on the response of HA1.7 (Fig. 3b). These results again indicated that DE-9 was more dependent on LFA-1 than HA1.7. The more general applicability of these results to CD4+ T cell populations of different functional phenotype was investigated by determining the co-stimulatory activity of LFA-1 on TH-1 and TH-2 CD4+ T cell lines. Anti-CD11a and anti-CD18 antibodies, co-immobilized with anti-CD3 antibodies, strongly enhanced the response of the *Der p* II line (TH-2) to anti-CD3 antibodies alone, whereas the response of the MTSE line (TH-1) was unaffected by the presence of the anti-LFA-1 antibodies (Fig. 4).

Activation and induction of non-responsiveness induces phenotypic modulation of accessory molecules in T cell clones

We have previously demonstrated phenotypic changes, including downregulation of CD3 and upregulation of CD25, in the T cell clone HA1.7 associated with activation [15]. Levels of CD11a, CD18 and CD54 on both HA1.7 and DE-9 were enhanced following activation by a combination of cross-linked anti-CD3 antibodies and IL-2 (Fig. 5). Exposure of CD4+ T cells to supraoptimal concentrations of peptide or superantigen in the absence of accessory cells induces non-responsiveness (anergy)

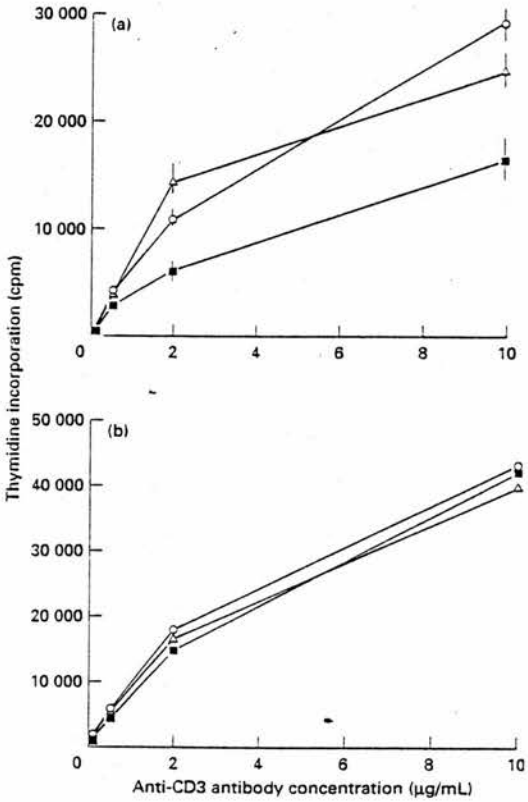

Fig. 4. Effect of cross-linked antibodies to CD11a/CD18 on the response of *Der p* II (a) and MTSE (b) specific T cell lines to anti-CD3 antibodies. Varying concentrations of anti-CD3 antibodies were co-immobilized with antibodies to CD11a or CD18 (10 μg/mL) on plastic wells. T cells (1×10^5/mL) were added to each well and proliferation measured as in Fig. 1. –■–. Anti-CD3. –○–. Anti-CD3 + anti-CD11a. –△–. Anti-CD3 + anti-CD18.

resulting in down-regulation of CD3 and increased CD25 expression in HA1.7 [15]. This analysis was extended to the changes in expression of adhesion molecules (Fig. 5). Anergizing concentrations of both peptide or superantigen induced upregulation of CD11a, CD18 and CD54 in both HA1.7 and DE-9. A parallel functional assay (Table 1) indicated that cloned T cells had indeed been anergised by exposure to both peptide and superantigen. In contrast, treatment with anti-CD3 antibodies and IL-2 does not impair the ability of the T cell clones to respond to immunogenic challenge (Table 1).

Table 1. Proliferative responses of CD4+ T cell clones. HA1.7 and DE-9

Treatment[a]	HA1.7	DE-9
Medium	14786(14)[b]	17435(24)
Anti-CD3 + IL-2	12657(21)	20564(15)
HA307	1546(9)	ND
SEB	2435(12)	ND
SEE	ND	3546(12)

[a] T cells were exposed for 16 h to the designated treatments (see Materials and methods). Cells were washed and restimulated with HA307 peptide (2 μg/mL) or *D. pter.* (10 μg/mL) in the presence of APC.
[b] Mean proliferative response of triplicates (%SEM).

Discussion

In this study we have compared the function of LFA-1 in the activation of human CD4+ T cells belonging to TH-0, TH-1 and TH-2 functional phenotypes. Immunogenic challenge of cloned CD4+ T cells representative of TH-0 and TH-2 phenotypes in the presence of soluble antibodies to CD11a and CD18 resulted in inhibition of T cell proliferation, presumably due to abrogation of interactions between LFA-1 and ICAM-1. House dust mite-reactive T cells of clone DE-9 were more susceptible than HA1.7 to inhibition, suggesting greater dependence on the accessory function of LFA-1. Although expression of CD11a and CD18 was higher in HA1.7 than DE-9, anti-LFA-1 antibody concentrations of 20 μg/mL are likely to be saturating. Previous experiments have shown that DE-9 is also more dependent than HA1.7 on the accessory molecule, CD4, during antigenic stimulation [16].

Although it has been reported that cross-linked anti-CD3 antibodies can induce proliferation in polyclonal CD4+ T cells [13], it is probable that after rigorous depletion of monocytes, a co-stimulatory signal is required to induce proliferation [14]. CD4+ T cells lines and clones, in contrast, proliferate to anti-CD3 antibodies in the absence of co-stimulation. Nevertheless, optimal stimulation of CD4+ T cell lines has also been reported to require co-stimulation through LFA-1 by the counter-receptors ICAM-1 and ICAM-2 [17]. T cells of the TH-2 clone, DE-9, were optimally stimulated only in the presence of antibodies to CD11a and CD18. The co-stimulatory activity transduced through LFA-1 may be a general property of TH-2 cells as a *Der p* II-specific CD4+ T cell line, drived from an atopic donor, also required signals through LFA-1 for optimal stimulation. In contrast, neither an MTSE-specific CD4+ T cell line of TH-1 functional phenotype or the TH-0 clone,

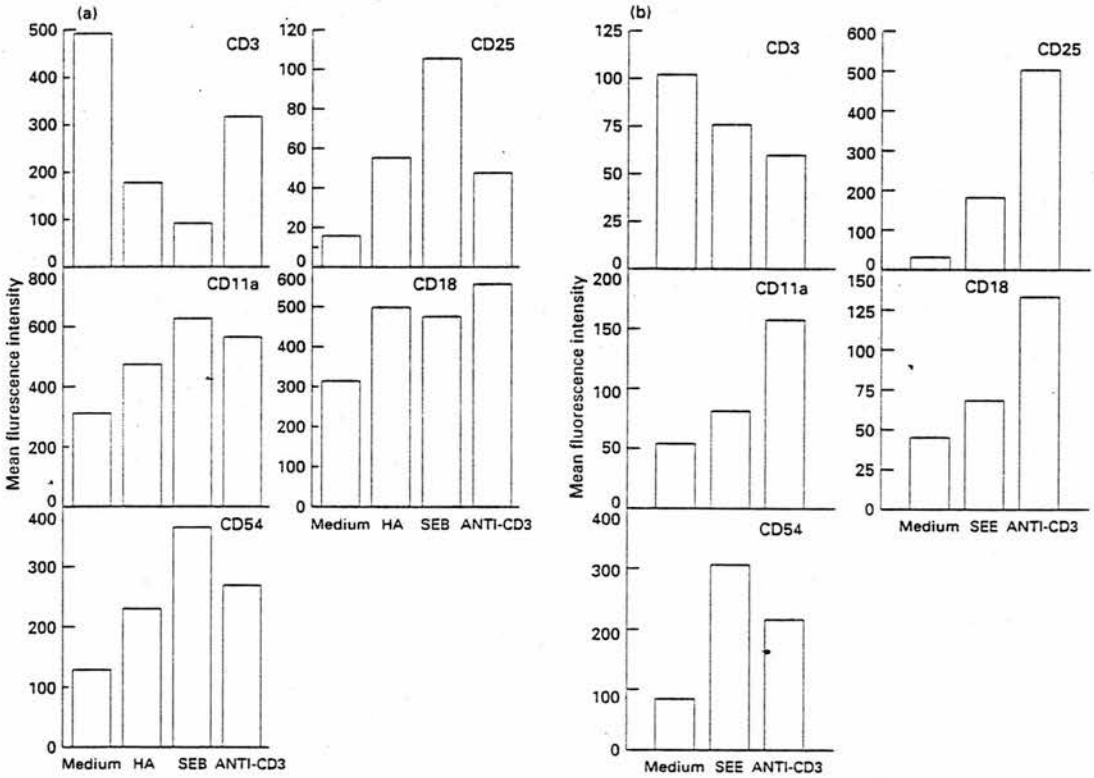

Fig. 5. Comparison of the effects of activation and induction of non-responsiveness (anergy) in HA1.7 and DE-9 on phenotypic modulation of adhesion molecules. The T cell clones (2×10^6/mL), HA1.7 (a) and DE-9 (b) were incubated in the presence of medium alone or anti-CD3 antibodies (10 μg/mL) together with IL-2 (5% Lympocult-T). Additional aliquots of HA1.7 were also incubated with HA307 (50 μg/mL) or SEB (0·5 μg/mL). An aliquot of DE-9 was also incubated with SEE (5 μg/mL). After overnight incubation each aliquot of cells was washed and phenotypic expression of CD3, CD25, CD11a, CD18 and CD54 determined by flow cytometry.

HA1.7, had any apparent requirement for co-stimulation through LFA-1. It is probable that antibodies to adhesion molecules increase adhesion and thus the affinity of interaction of co-immobilized anti-CD3 antibodies with the TCR. The observation that anti-CD11a and anti-CD18 antibodies provided similar levels of co-stimulation to the *Der p* II-specific T cell line and DE-9 would support this postulate. A purified ICAM-1 antigen has recently been reported to provide co-stimulation to polyclonal CD4+ T cells by prolonging inositol phospholipid hydrolysis [7], an important signal transduction pathway in the activation of human CD4+ T cells. Inositol phospholipid hydrolysis may be optimally stimulated in TH-0 or TH-1 CD4+ T cells by anti-CD3 antibodies in the absence of accessory signals. Alternatively, it is possible that ligand binding of LFA-1 antibodies may not completely reflect the binding of ICAM. It is possible that different T cell subsets have differential dependence on the three ICAM molecules [18]. Another possibility is that TH-0 and TH-1 cells may transduce signals through adhesion molecules other than LFA-1. Murine TH-1 cells have been shown to use different signalling pathways to TH-2 cells [19].

In contrast to the effects of co-immobilized anti-CD18 antibodies, soluble antibodies to CD18 inhibited the response of DE-9. Soluble anti-CD18 antibodies have previously been shown to inhibit anti-CD3 mediated stimulation of polyclonal T cells [13]. Soluble

anti-CD11a antibodies had no effect on the response and therefore, anti-CD18 antibodies were not inhibiting interactions between opposing T cells. A negative signal appeared to be transduced specifically through CD18. A strong intracellular calcium flux was induced following ligation of CD18 on polyclonal CD4+ T cells by 60.3, the anti-CD18 antibody used in this study [20], and this may be associated with a negative signal to the TCR. It seems probable that co-immobilization more accurately reflects the distribution of MHC class II/peptide complexes and adhesion molecules on the surface of the accessory cell and, therefore, that a co-stimulatory positive signal by adhesion molecules will normally result from immunogenic challenge. Signal transduction through the TCR is known to involve a sequence of coordinated biochemical reactions transduced through the TCR multimeric complex [21]. Although there is no evidence of a physical association of LFA-1 with the TCR, the observed upregulation of CD11a, CD18 and CD54 after stimulation of HA1.7 and DE-9 indicates that these molecules are linked to TCR signalling.

Increased expression of adhesion molecules on allergen-specific CD4+ T cells is related to recirculation and extravasation of lymphocytes through endothelial cells into tissue sites. The predominant T cell infiltrate in skin sites sensitized to grass pollen allergens appeared to be of TH-2 phenotype [22]. Desensitization to HDM allergens might be attained by functionally inactivating, or anergising, HDM-specific CD4+ T cells of the TH-2 functional phenotype. Anergized HDM-specific CD4+ T cells express high levels of LFA-1/ICAM-1 making it probable that they could also extravasate into tissue sites. The functional inactivation of peptide desensitized HDM-specific CD4+ T cells is not total since on immunogenic challenge IFNγ production is maintained, although IL-4 secretion is ablated [23]. Release of IFNγ by these cells might act to regulate and inhibit TH-2 cytokine production by other HDM-specific CD4+ T cells [24].

Acknowledgements

We would like to thank Dr N. Hogg, ICRF, London, for information and advice on adhesion molecules. The support of the Wellcome Trust and the Medical Research Council is gratefully acknowledged. R. O'H. is a Wellcome Senior Clinical Fellow.

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Clinical and Experimental Allergy, 1995, Volume 25, pages 839–847

Clonal analysis of CD4 mediated accessory function on the effector activity of human CD4+ T cell subsets

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Summary

Background It has been reported for the peripheral T cell repertoire that CD4 molecules may enhance adhesion between T cells and antigen presenting cells and, through their physical association with T cell antigen receptors, contribute to signal transduction.

Objective The aims of this study were to determine if the modulation of CD4 molecules had differential effects on T cell recognition, antigen induced cytokine (IL-4 and IFNγ), release and the induction of specific anergy for human TH-0, TH-1 and TH-2 cells.

Methods A panel of anti-CD4 antibodies was examined for its ability to modulate T cell proliferation, cytokine production and tolerance induction in house dust mite (TH-0 and TH-2) and influenza haemagglutinin (TH-1) specific human CD4+ T cell clones all restricted by DRB1*1101 and isolated from dust mite allergic individuals.

Results We observed that anti-CD4 antibodies may inhibit or enhance antigen mediated T cell proliferation, which may reflect the differential requirements of T cells for selective functions of CD4. Furthermore, IFNγ and IL-4 production was differentially modulated depending on the specificity of the anti-CD4 antibody and the clone of T cells. However, pretreatment of T cells with anti-CD4 antibody alone neither induced nor enhanced the susceptibility of T cells to peptide mediated anergy.

Conclusion Antigen recognition by different subsets of human CD4+ T cells has differential requirements on CD4, whereas the induction of specific anergy appeared to be independent of the functions of CD4 molecules. Antigen induced IFNγ production was more susceptible than IL-4 to the inhibitory effects of anti-CD4 antibodies. Furthermore, it appeared that certain anti-CD4 antibodies can dissociate antigen induced IFNγ and IL-4 production, and may downregulate IFNγ synthesis without inhibiting antigen dependent proliferation.

Keywords: CD4, CD4+T cells subsets, house dust mite, haemagglutinin, cytokines, T cell recognition, anergy

Clinical and Experimental Allergy, Vol. 25, pp. 839–847. Submitted 12 December 1994; revised 7 March 1995; accepted 30 March 1995.

Introduction

Different CD4+ T subsets, termed TH-1 and TH-2, promote either cell-mediated or humoral immunity respectively, illustrating the functional heterogeneity of the CD4+ T cell repertoire [1]. This polarity in activity is determined by the cytokines produced by each of CD4+ T cell subsets, with TH-2 cells characteristically secreting (interleukin) IL-4, IL-5 and IL-6, whereas TH-1 cells secrete IL-2 and interferon-gamma (IFNγ) [reviewed in ref. 2]. An intermediate subset, TH-0 cells, which secrete

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both TH-1 and TH-2 cytokines have been identified [3] and may form the major functional component of the peripheral human T cell repertoire [4]. In addition kinetics and quantitative differences in the cytokine response profiles will also contribute to the heterogeneity of the CD4+ T cell responses.

The induction of protective or pathogenic immunity is determined, in part, by which CD4+ T cell subsets dominate in the response to challenge with specific antigens [5,6]. However, the factors which determine the selective activation of these distinct immune pathways have not been fully defined. There is evidence to suggest that, in addition to the local cytokine environment [7,8], the affinity of T cell antigen receptor (TcR) [9,10], MHC class II and peptide interactions may influence the functional phenotype of the CD4+ T cells that are activated [11,12], with weak affinity interactions and low antigen density favouring the induction of TH-2 cells. When antigen is limiting or the TcR has a low affinity for its ligand the interaction of accessory molecules with their counter receptors on antigen presenting cells (APC), such as CD4 with MHC class II molecules, may increase the affinity of TcR for peptide/MHC class II complexes. Therefore, activation of T cells with low affinity TcR may depend more upon the accessory function of CD4 mediating increased adhesion [13,14].

The role of CD4+ molecules on T cell effector function extends beyond facilitating cell adhesion and contributes to antigen recognition and T cell activation. Comodulation of CD4 and TcR molecules on selected T cells with anti-TcR antibodies or antigen implies that CD4 may be a structural component of the antigen recognition complex on T cells [15,16]. The extracellular domains of CD4 may serve to correct for variations in the flanking regions of naturally processed peptides bound to MHC class II molecules [17]. Physical association of CD4 with the TcR and consequent provision of tyrosine kinase activity by the CD4 associated $p56^{lck}$ may be required for optimal antigenic stimulation [18]. Evidence that negative as well as positive signals can be delivered through CD4 has come from studies on the binding of the CD4 ligand, HIV-gp120 [19]. The administration of non-depleting anti-CD4 antibodies together with antigen in mice results in the induction of specific tolerance [reviewed in ref. 20], demonstrating that the manipulation of CD4 may also result in the downregulation of T cell responses.

Collectively these studies prompted us to investigate the influence of CD4 molecules on antigen recognition, cytokine production and the induction specific anergy by CD4+ human T cell clones respresentative of the TH-0, TH-1 and TH-2 subsets. Our results demonstrate that effects of anti-CD4 antibodies on antigen dependent T cell responses are heterogeneous. They may either inhibit or enhance proliferation, but fail to induce or to modulate peptide-mediated T cell anergy. We observe that IFNγ production is more susceptible than that of IL-4 to the inhibitory effects of anti-CD4 antibodies. Furthermore, anti-CD4 mediated downregulation of IFNγ production may occur without inhibition of antigen dependent proliferation.

Materials and methods

Antibodies and antigens

Purified murine monoclonal IgG antibodies (ADP 372 (D4003), ADP 318 (Q4120) and ADP 302 (RFT4) specific for different epitopes of human CD4 were obtained from the MRC AIDS Directed Programme. The anti-CD4 antibody L200 was the kind of gift of Dr G. Fathman, Department of Immunology and Microbiology, Stanford Medical Center, Palo Alto, CA, USA. The anti-HLA class I antibody (W6/32) was used as a control. The amount of antibody required to maximally stain CD4+ T cell clones as assessed by FACS analysis has been used as the initial starting concentration in the functional assays and is termed 1 : 100 dilution.

The *Der p* II peptides spanning residues 28–40 and HA peptide (255–270) were synthesized by standard solid phase methods as previously described [21] on an Applied Biosystem 431A synthesizer. Lyophilized unfractionated extracts of *Dermatophagoides pteronyssinus* (house dust mite; HDM) were generously provided by Dr A. Wheeler, SmithKline and Beecham, UK. Purified influenza virus haemagglutinin (HA) from the recombinant X31 virus was kindly provided by Dr J. Skehel, NIMR, Mill Hill, London, UK.

Subjects and separation of peripheral blood mononuclear leucocytes

Both the HDM and HA reactive T cell clones used in this study were isolated from the peripheral blood of dust mite allergic individuals. Feeder cells required for the maintenance and expansion of the T cell clones were isolated from non-atopic control subjects. Atopic status and house dust mite allergy were defined by positive skin-prick tests of $\geqslant 3$ mm diameter weal to the standard panel of common environmental aeroallergens in the presence of a positive histamine and negative saline control, together with the presence of HDM specific serum IgE antibodies and clinical history.

Peripheral venous blood was collected in preservative free heparin (CP Pharmaceuticals Ltd, Wrexham, UK). Mononuclear cells (PBMC) were separated by density gradient centrifugation on Ficoll-Hypaque (Pharmacia,

UK) and resuspended in complete medium (RPMI 1640 supplemented with 2 mM L-glutamine, 100 IU/mL penicillin/streptomycin and 5% screened, heat inactivated human A+ serum) and used in proliferation assays.

Isolation and characterization of T cell clones

The HDM reactive (*Der p* II) and HA reactive T cell clones were isolated from HDM reactive individuals by limiting dilution cloning [21,22]. Briefly, PBMC (10^5/mL) were stimulated with an optimal concentration of antigen (10 μg/mL) for 7 days in complete medium. A long-term line was established by enriching lymphoblasts on Ficoll-Paque (Pharmacia) in the presence of irradiated autologous PBMC (5×10^5/mL; 2500 Rads), antigen (10 μg/mL) and interleukin 2 (IL-2, 5% v/v; Lymphocult T, Biotest Folex, Frankfurt, Germany). Cells from the T cell line (0·3 cells/well) were cloned by limiting dilution in complete medium and plated in Microtest II plates together with irradiated autologous PBMC, antigen and IL-2. After 7 days, growing T cell clones were transferred to flat-bottom 96 well microtitre plates and then to 24 well plates. The T cell clones were maintained with IL-2 every 7 days and were used in experiments 7–8 days after the last addition of antigen and APCs. The T cell clones AC1 and AC2 recognize residues 25–40 of the group II allergen (*Der p* II) of HDM and the T cell clone, PF5, was reactive with residues 255–270 of HA. All three T cell clones recognize antigen in association with HLA-DRB1*1101 class II molecules.

Proliferation assays

T cell clones (10^5/mL) were cultured with peptides (*Der p* II, 3 μg/mL; HA, 1 μg/mL) in the presence of irradiated autologous PBMC ($1{\cdot}25 \times 10^6$/mL; 2500 Rads) and increasing dilutions of antibody. After 48 h the cultures were pulsed with tritiated methyl thymidine ([^{3}H]-TdR; 1 μCi/well, [^{3}H]-TdR; Amersham International plc, Amersham, UK) and harvested 8–16 h later. Proliferation as measured by [^{3}H]-TdR incorporation was determined by liquid scintillation spectroscopy. The results are expressed as mean cpm for triplicate cultures with SEM< 25%.

Induction of T cell anergy

T cell clones (AC1 and PF5; 10^6/mL) were pretreated with anti-CD4 antibody (L200 at 1 : 100 dilution), anti-MHC class I antibody (W6/32) or medium for 1 h at 37°C and then cultured in the presence of increasing concentrations of peptide for 18 h as previously described [23]. The T cells were then washed extensively and restimulated with either an optimal concentration of peptide in the presence of APCs or IL-2 alone and proliferation measured by the incorporation of 3[H]-TdR.

Cytokine assays

T cell clones (10^5/mL) were stimulated with and without peptides in the presence of irradiated autologous PBMC ($1{\cdot}25 \times 10^6$/mL; 2500 Rads), either alone or together with antibody. The supernatants from these cultures were collected at 24 h for the determination of cytokine levels. IFNγ and IL-4 were measured in ELISA assays (Eurogenetics, Teddington, UK).

Results

Modulation of antigen dependent T cell proliferation by anti-CD4 antibodies of human T cell clones representative of the TH-0, TH-1 and TH-2 functional phenotypes

The functional characteristics of the human T cell clones used in this study are summarized in Table 1. The T cell clones AC1 and AC2 both reactive with *Der p* II residues 25–40 and restricted by DRB1*1101 have the cytokine profiles of the TH-0 and TH-2 functional phenotypes respectively. PF5 reactive with influenza virus HA residues 255–270 and restricted by DRB1*1101 secretes IFNγ but undetectable levels of IL-4 and, therefore, is designated in the TH-1 subset. The T cells were stimulated

Table 1. Functional characterisation of T cell clones

	AC1	AC2	PF5
TH-0/TH-1/TH-2	TH0	TH2	TH1
MHC class II restriction	DRB1*1101	DRB1*1101	DRB1*1101
Antigen specificity	*Der p* II, 25–40	*Der p* II, 25–40	HA, 255–270

with specific antigen and APCs in the presence and absence of anti-CD4 antibodies (Fig. 1 a–c). Antigen mediated proliferation of the T cell clone AC1 was inhibited in a dose dependent manner by the anti-CD4 antibodies 302, 318 and L200, although they differed in potency (Fig. 1a). The control anti-HLA class I antibody W632 had minimal inhibitory effects on the response of AC1 to antigen. In the presence of the same panel of anti-CD4 antibodies a similar pattern of inhibition of antigen dependent proliferation was observed for T cells of AC2, with L200 being the most effective antibody (Fig. 1b). Again no inhibition was induced by the anti-class I antibody. In contrast, the anti-CD4 antibodies failed to inhibit the response of the HA reactive T cells, PF5 to antigen (Fig. 1c). Indeed, the anti-CD4 antibody 318 enhanced the antigen specific proliferation. The other antibodies had neither inhibitory nor stimulatory effects on the response of the PF5 T cells (Fig. 1c). These results indicate heterogeneity in the effects of anti-CD4 antibodies on antigen mediated proliferation of different CD4+ T cells, which may reflect the functional phenotype of the T cells or variable affinities of the HDM and HA peptides for binding to DRB1*1101 class II molecules.

Effect of anti-CD4 antibodies on cytokine production by cloned TH-0, TH-1 and TH-2 cells

Supernatants were collected from T cell clones AC1, AC2 and PF5 stimulated with specific antigen and APCs in the presence of anti-CD4 antibodies and the levels of IL-4 and IFNγ determined (Figs 2–4). In the presence of the anti-CD4 antibodies (318 and L200) both IL-4 and IFNγ production by the TH-0 cells (AC1) was inhibited in a dose dependent manner (Figs 2a and c). However, although able to inhibit antigen dependent IFNγ production, the anti-CD4 antibody 302 (Fig. 2b) failed to downregulate IL-4 secretion. No modulation of either IL-4 or IFNγ production was observed in the presence of the control antibody W632 (Fig. 2d). In the presence of the panel of anti-CD4 antibodies IL-4 production by AC2 (TH-2) was inhibited by the antibodies 318 and L200 but not 302 or W6/32 (Fig. 3).

IFNγ secretion by the influenza HA reactive TH-1 clone PF5 was reduced by the addition of the antibodies 318 or L200 (Fig. 4). No modulation of cytokine

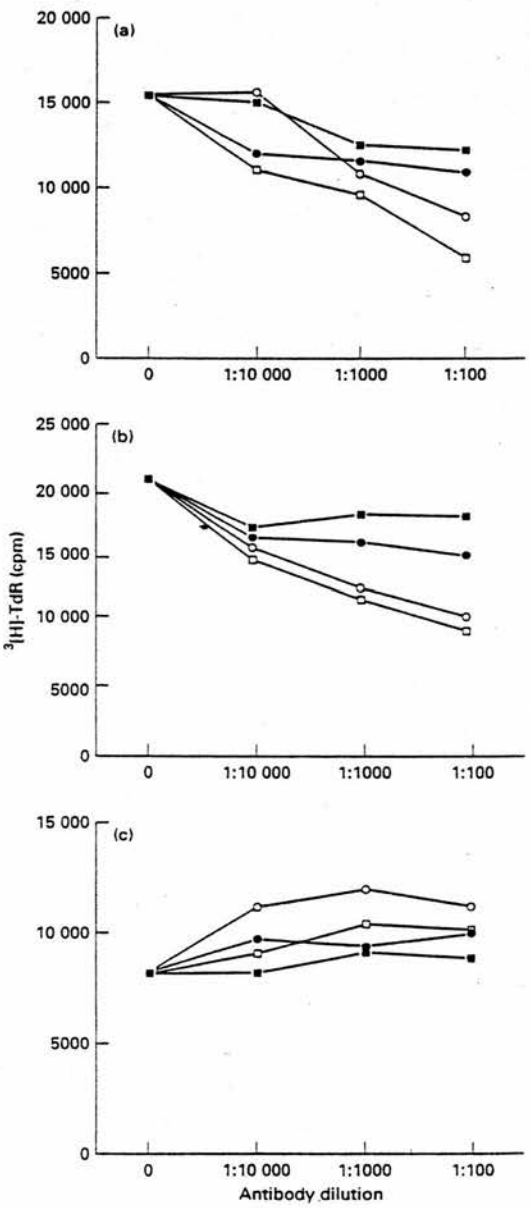

Fig. 1. Modulation of antigen dependent proliferation of human TH-0, TH-1 and TH-2 cell clones by anti-CD4 antibodies. T cell clones were stimulated with specific peptide and APCs (EBV-B cells) in the presence and absence of a panel of anti-CD4 (302, ●; 318, ○; L200, □) and anti-HLA class I (W632, ■) antibodies at different dilutions. 3[H]-TdR incorporation was measured at 72 h. Results are expressed as mean of triplicate cultures. In all experiments SEM<25%. (a) AC1. (b) AC2. (c) PF5.

production was observed when 302 or W6/32 were added to the cultures (Fig. 4).

The failure of anti-CD4 antibodies to induce, enhance or prevent the induction of peptide-mediated T cell anergy

In the presence of antigen, non-depleting anti-CD4 antibodies will induce specific tolerance *in vivo* [20]. Therefore, we investigated if signalling via CD4 alone will induce or enhance peptide-mediated T cell anergy. T cells of clones AC1 and PF5 were cultured with L200 alone or in the presence of increasing concentrations of peptide and the response to rechallenge with peptide and APCs or IL-2 determined. The proliferative responses of control cultures of cells exposed to peptide alone or peptide in the presence of W6/32 were compared (Fig. 5). Pretreatment of AC1 and PF5 with medium or antibodies (anti-CD4 or anti-MHC class I) alone in the absence of peptide failed to induce T cell anergy in that the T cells still responded when restimulated with peptide and APCs (Figs 5a and c). Furthermore, exposure of AC1 to high doses of peptide resulted in the induction of anergy that was neither enhanced nor inhibited by the addition of anti-CD4 antibody (Fig. 5a). The TH-1 cells (PF5) were also rendered anergic by high antigen concentrations in a similar fashion, although the threshold of peptide required to induce anergy was lower than that observed for AC1 (Fig. 5c). Enhanced responsiveness to IL-2, characteristic of anergic T cells, was observed in all the groups of T cells (Figs 5b and d).

Discussion

In this study we have investigated the contribution of CD4 molecules in antigen recognition and effector function of human T cell clones representative of the TH-0, TH-1 and TH-2 functional phenotypes. Previous studies have reported that inhibition of the effector function of CD4+ T cells with anti-CD4 antibodies correlates with the avidity of the interaction between the T cells and

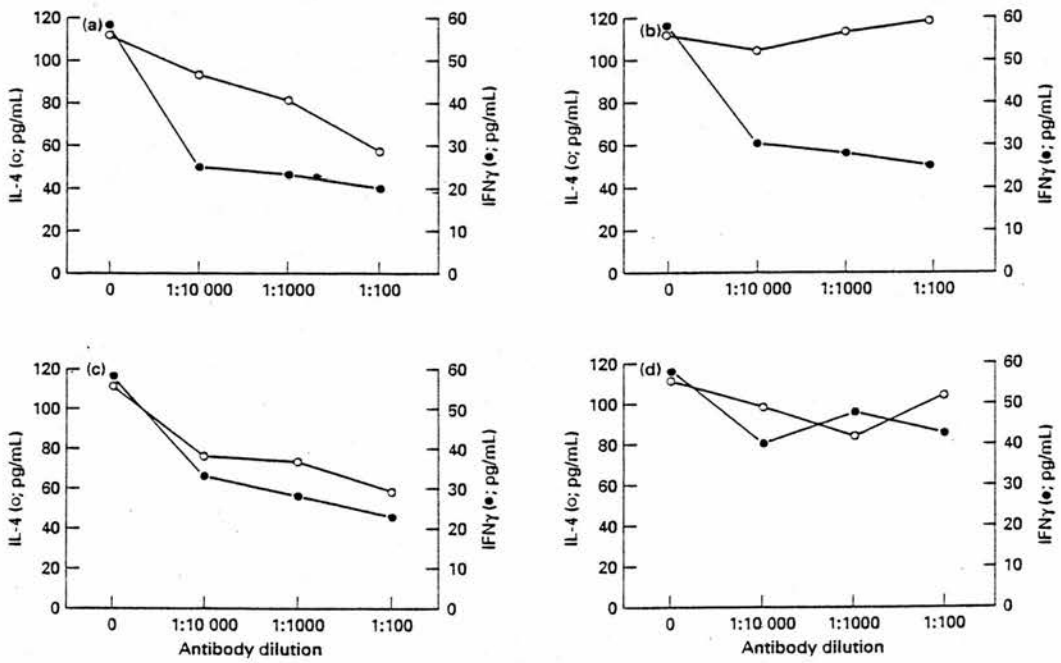

Fig. 2. Effect of anti-CD4 antibodies on antigen mediated IL-4 and IFNγ production by cloned TH-0 cells. T cells (clone AC1) were stimulated under the conditions described in the legend to Fig. 1. Supernatants were collected at 24 h and the levels of IL-4 (O) and IFNγ (●) measured by ELISA. In the absence of antigen T cells cultured with APCs and antibody produced undetectable levels of IL-4 or IFNγ.

APCs [13,14]. The avidity of the interaction appears to be influenced by the orientation of the TcR thus determining its capacity to associate with CD4 [24]. Using anti-CD4 antibodies specific for different epitopes on CD4 we observed that the same pattern of inhibition of antigen proliferation was induced in both clones of DRB1*1101 restricted T cells specific for *Der P* II, residues 25–40 [21]. However, despite having the functional characteristics of either the TH-0 or TH-2 phenotypes there was no apparent difference in their dependency on CD4 accessory function. The loss of antigen mediated proliferation may result from disrupting the function of CD4 as an adhesion molecule and may occur by preventing the formation of complexes between TcR, MHC class II and peptide. The ability of CD4 to stabilize class II molecules in dimeric form and increase the density of MHC class II/peptide complexes may be inhibited in the presence of the anti-CD4 antibodies. Alternatively, the inhibitory effects of the anti-CD4 antibodies may arise from the delivery of a negative signal directly to the T cells, since it has been reported that crosslinking of CD4 molecules with specific monoclonal antibodies before activation through TcR results in the loss of T cell responses [26]. Contrary to these results we observed that pretreatment of the T cell clones with the anti-CD4 antibodies had no inhibitory effects on antigen recognition. The role of CD4 in signal transduction is indicated by the enhancement of antigen induced proliferation by the influenza HA reactive TH-1 cells following the addition of the anti-CD4 antibodies, 318 and L200. Earlier studies have established that crosslinking both CD4 and TcR/CD3 with antibodies may induce synergistic proliferative responses [15,27] and one explanation offered for this observation is that the anti-CD4 antibodies are stabilizing the interaction between CD4 and the TcR complex. Orientation of the different chains of the TcR determines the association with CD4 [24].

The different effects of CD4 on antigen induced proliferation by the TH-0, TH-1 and TH-2 clones studied here prompted us to investigate the effect of the modulation of CD4 on the cytokine response profiles. The addition of anti-CD4 antibodies, 318 and L200, to antigen stimulated cultures of the TH-1 cells inhibited both IL-4 and IFNγ production. However, the anti-CD4 antibody 302, although able to downregulate IFNγ syntheses failed to inhibit IL-4 production. Likewise, *Der p* II dependent IL-4 production by the TH-2 cells (AC2) was not inhibited by the antibody 302, whereas the other anti-CD4 antibodies downregulated IL-4 release. Although capable of enhancing the proliferative

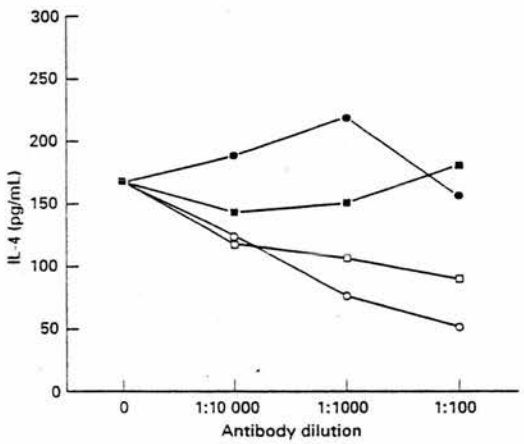

Fig. 3. Effect of anti-CD4 antibodies on antigen mediated IL-4 production by cloned TH-2 cells. T cells (clone AC2) were stimulated with anti-CD4 (302, ●; 318, ○; L200, □) and anti-HLA class I (W6/32, ■) antibodies under the conditions described in the legend to Fig. 1. Supernatants were collected at 24 h and the levels of IL-4 measured by ELISA. In the absence of antigen T cells cultured with APCs and antibody produced undetectable levels of IL-4.

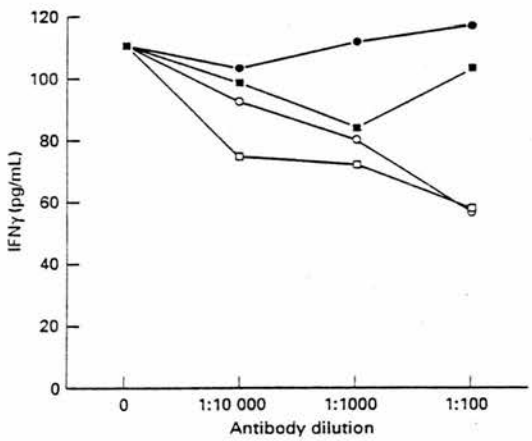

Fig. 4. Effect of anti-CD4 antibodies on antigen mediated IFNγ production by cloned TH-1 cells. T cells (clone PF5) were stimulated with anti-CD4 (302, ●; 318, ○; L200, □) and anti-HLA class I (W6/32, ■) antibodies under the conditions described in the legend to Fig. 1. Supernatants were collected at 24 h and the levels of IFNγ measured by ELISA. In the absence of antigen T cells cultured with APCs and antibody produced 19 pg/mL of IFNγ.

response of the TH-1 clone (PF5), presumably by inducing IL-2 secretion, the antibodies L200 and 318 inhibited IFNγ production. Our observations suggest that certain anti-CD4 antibodies have variable effects on T cell recognition and antigen dependent cytokine production by different CD4+ T cell subsets. In some instances the effector functions determined by differences in the biological activity of the cytokines they produce, such as IL-4 and IFNγ, can be dissociated by selectively regulating or inhibiting their synthesis. Depending on which cytokines dominate the immune response they may determine whether the immune response to antigen is protective or pathogenic. For example, exposure of atopic individuals to environmental allergens induces TH-2 cells and the formation of allergen specific IgE, while in non-atopic subjects, T cell responses of TH-1 phenotype generate no pathological changes [5,6]. Therefore, the ability to inhibit IL-4 production or promote IFNγ synthesis through the action of anti-CD4 antibodies may result in reduced specific IgE levels and so contribute to the treatment of allergic diseases. The anti-CD4 antibodies may mediate their differential effects on cytokine production by altering the affinity of the interaction of the TcRs with MHC class II/peptide complexes and subsequently changing the threshold of signalling via the TcR [17,28]. Studies on murine CD4+ T cell clones have demonstrated that different dose thresholds are required for distinct effector function [29]. It is possible that similar changes in the threshold of signalling and selective regulation of cytokine release by anti-CD4

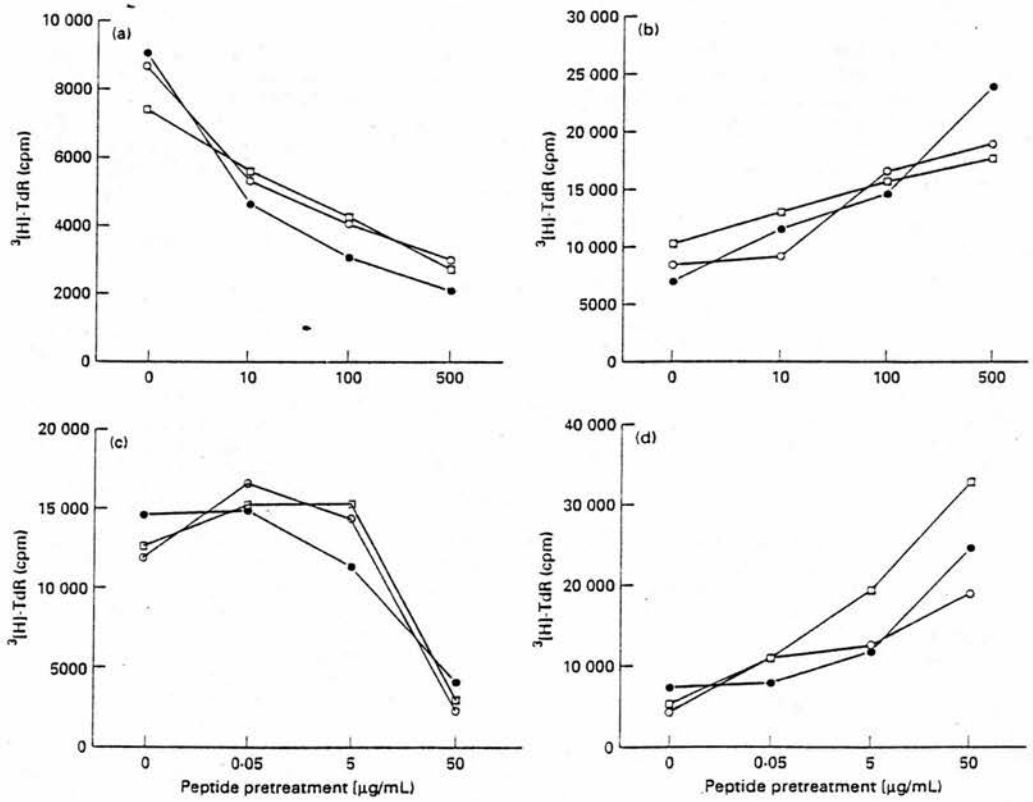

Fig. 5. Anti-CD4 antibodies fail to induce or prevent the induction of mediated T cell anergy. Cloned T cells (AC1 and PF5) were cultured in medium (○), anti-CD4 (L200, ●) or anti-HLA class I (W6/32, □) antibodies alone or in the presence of increasing concentrations of specific peptide. After overnight incubation the T cells were restimulated with optimal concentrations of peptide (*Der p* II or HA) in the presence of APCs (A, AC1; C, PF5) or IL-2 alone (B, AC1; D, PF5) and 3[H]-TdR measured.

antibodies may contribute to the mechanisms by which 'non-depeleting' anti-CD4 antibodies in the presence of specific antigen are able to induce infectious intolerance *in vivo* [20].

The exposure of human CD4+ T cells to supraoptimal concentrations of peptide in the absence of appropriate costimulatory signals results in the induction of T cell anergy, which is characterized by the loss of antigen dependent proliferation, phenotypic modulation and cytokine dysregulation [30]. Anti-CD4 antibodies, in the presence of specific antigen, will induce tolerance *in vivo* and this prompted us to examine the role of CD4 in the induction of peptide-mediated anergy in human T cells. We observed that crosslinking CD4 neither induced anergy nor reduced the threshold of peptide required for the state of anergy to be achieved in contrast to the effects of HIV-gp120 [19] and this may reflect the use of different signalling pathways. In these experiments the capacity of CD4 to modulate anergy was investigated using fully differentiated T cells, however, naive CD4+ T cell populations may be more susceptible to manipulation.

The relevance of our findings in relation to allergic disease suggest that certain anti-CD4 antibodies can induce selective regulation of cytokines and in some cases have the ability to downregulate IL-4 production.

Acknowledgments

This work was supported by grants from the Wellcome Trust and the Medical Research Council, UK. DNAX Research Institute is supported by Schering Plough. R. E. O'Hehir is the recipient of a Wellcome Senior Research Fellowship in Clinical Sciences.

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2.3 ANTIGEN MEDIATED MODULATION OF T CELL FUNCTION

2.3.1 INHIBITION OF T CELL PROLIFERATION

O'Hehir RE et al: *J Allergy Clin Immunol* 1991, **87**:1120-1127.

Hawrylowicz CM et al: *J Allergy Clin Immunol* 1996, **97**:707-709.

Reprinted from THE JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY, St. Louis
Vol. 87, No. 6, pp. 1120-1127, June, 1991. (Printed in the U.S.A.)

An in vitro model of peptide-mediated immunomodulation of the human T cell response to *Dermatophagoides* spp (house dust mite)

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Jonathan B. Rothbard, PhD, and Jonathan R. Lamb, PhD**
** London, England, and Palo Alto, Calif.*

Allergic sensitivity of Dermatophagoides *spp (house dust mites) is mediated by specific IgE antibody, the production of which requires the presence of CD4⁺ helper T cells. Attempts to hyposensitize this response in allergic individuals have depended on the administration of extracts of specific allergen. However, the ability of peptides derived from unrelated antigens to inhibit specific immune responses offers an alternative approach to therapy. We have addressed this question by examining the ability of a nonstimulatory peptide analogue derived from influenza virus hemagglutinin to modulate T cell recognition of house dust mite. The peptide inhibited the response of mite-specific CD4⁺ T cell clones restricted by either the HLA-DRAB1 or DRAB3 gene products. Furthermore, mite-induced polyclonal T cell responses were negatively modulated by the peptide, whereas recognition of common recall antigens remained intact. The inhibitory effects were mediated at the level of the antigen-presenting cell, since no inhibition of mitogen or anti-CD3 antibody-driven T cell proliferation was observed. In direct binding assays, the peptide analogue bound to selected HLA-DR molecules expressed on the membrane of antigen-presenting cells, with specificity predominantly for those class II proteins capable of restricting house dust mite-allergen T cell recognition. (J ALLERGY CLIN IMMUNOL 1991;87:1120-7.)*

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Supported by the Wellcome Trust, England, and the Medical Research Council, England.
Received for publication July 12, 1990.
Revised Nov. 16, 1990.
Accepted for publication Dec. 20, 1990.
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*R. E. O'Hehir, MD, is the recipient of a Wellcome Senior Research Fellowship in Clinical Science.
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The importance of CD4+ T-lymphocytes in the pathogenesis of allergic diseases is clearly established with the synthesis of specific IgE and the differentiation and growth of effector cells, such as mast cells and eosinophils being dependent on CD4+ T cells and their products.[1-6] The activation of CD4+ T cells is dependent on antigen receptors on T cells recognizing peptidic fragments of foreign proteins in association with self-gene products of the MHC expressed on the surface membranes of antigen-presenting cells.[7-9] It has been proposed that the ability of MHC class II molecules to bind only selected peptide fragments of antigens in part determines immune responsiveness.[10] The recent results of functional experiments and direct binding assays suggest that gene products of specific MHC class II molecules are capable of binding a large number of different peptides, and, similarly, a single peptide may bind to more than one MHC class II protein.[11-16] Despite the apparent permissiveness in binding, variation in class II MHC molecules may regulate T cell recognition by influencing both peptide binding and interaction with T cell receptors.[7,9,16] The preferential binding and capacity of peptides to compete one with another for occupancy of the antigen-combining site of MHC class II proteins[17-19] introduce the potential of nonstimulatory peptides as immunoregulatory molecules in the treatment of allergic diseases. Clearly, if competitive inhibition were to have a role in the regulation of allergic immune responses, then specificity of the blocking peptide for the particular allergen-associated MHC class II alleles would be necessary.

The aim of this study was to investigate the ability of a nonallergen-derived, nonstimulatory peptide to modulate the response of T cells from atopic individuals with the commonly observed allergy to HDMs (*Dermatophagoides* spp). Analysis of the MHC class II restriction specificity has suggested that HLA-DRAB3 gene products (DRw52b, the supertypic specificity) were the major restriction elements, rather than the classical HLA-DR specificities encoded by the DRAB1 genes.[20,21] A peptide analogue derived from the natural sequence of the carboxyl terminus of the HA-1 molecule of influenza HA (residues 307 to 319 with the naturally occurring tyrosine residue at position 309 substituted by serine) was demonstrated to be able to bind to selected class II molecules and inhibit both polyclonal and monoclonal T cell responses to HDM.

Abbreviations used

MHC:	Major histocompatibility complex
HA:	Hemagglutinin
IL-2:	Interleukin-2
HDM:	House dust mite
PBMC:	Peripheral blood mononuclear cell
[^{3}H]TdR:	Tritiated methylthymidine

METHODS

Antigens

Lyophilized extracts of *D. farinae* and *D. pteronyssinus* were the generous gifts of Drs. H. Løwenstein and C. Schou (ALK, Horsholm, Denmark) and Bencard (Brentford, Middlesex, U.K.), respectively. A soluble extract of *Mycobacterium tuberculosis* (MTSE) was generously provided by Dr. A. Rees (Medical Research Council Tuberculosis Unit, Hammersmith Hospital, U.K.). The HA peptide analogue (residues 307 to 319; serine at 309) and the keratin peptide (residues 1 to 9) were synthesized with solid-phase techniques[22] on an Applied Biosystems (Foster City, Calif.) peptide synthesizer with Pam resins, *N*-t-Boc-protected amino acids, and commercially available reagents (Applied Biosystems). The T cell mitogen PHA-P was purchased from Sigma Chemical Co. (St. Louis, Mo.), and the murine monoclonal anti-CD3 antibody (OKT3), purified from the hybridoma cell line, was purchased from the American Type Culture Collection (Rockville, Md.).

Preparation of lymphocytes and donor characterization

PBMCs obtained from unmedicated atopic adults with symptomatic HDM allergy (perennial rhinitis) were isolated by centrifugation on a discontinuous gradient of Ficoll-Paque (Pharmacia Ltd., Milton Keynes, U.K.). All subjects had positive skin prick tests to *Dermatophagoides* spp, and positive HDM RASTs. PBMCs were resuspended in complete medium, RPMI 1640 supplemented with A+ or AB+ serum, 2 mmol/L of L-glutamine, and 100 IU/ml of penicillin/streptomycin.

Isolation of antigen-reactive T cell clones

HDM-specific T cell clones were isolated as described previously.[23] Briefly, PBMCs (2.5×10^5/ml) were stimulated with an optimal concentration of *D. farinae* for 7 days in complete medium. Lymphoblasts enriched on Ficoll-Paque were maintained as a long-term line in the presence of irradiated (2500 rad) autologous PBMCs, *D. farinae*, and IL-2 (10% vol/vol; Lymphocult-T, Biotest Folex, Frankfurt, Germany) and then cloned by limiting dilution from the T cell line. For cloning, viable cells (0.3 cells per well) were plated in Microtest II (Nunclon; Nunc, Roskilde, Denmark) trays together with irradiated autologous PBMCs (5×10^5/ml), *D. farinae*, and IL-2. After 7 days, growing clones were transferred to flat-bottom, 96-well microtiter trays and, subsequently, to 24-well trays. At each transfer the clones received filler cells, antigen, and IL-2. The clones were maintained and expanded by the addition of IL-2 every 3 to 4 days and antigen together with filler cells every 7 days. Before their use in proliferation assays, the T cell clones were rested for 6 to 8 days after the last addition of filler

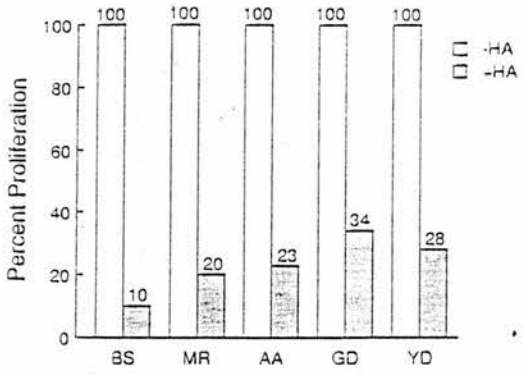

FIG. 1. Inhibition of polyclonal T cell response of dust mite-allergic individuals to specific allergen. PBMCs were stimulated with HDM in the presence *(stippled bar)* or absence *(open bar)* of the HA peptide analogue (residues 307-319 and S-309), both proteins being administered over a concentration range. Proliferation as correlated with [^{3}H]TdR incorporation was measured at day 7, and in all experiments, SEM for triplicate cultures was <25%. Results presented are the percentage proliferation as compared to the maximal HDM response expressed as 100%.

cells and antigen. A number of T cell clones specific for *D. farinae* and other clones cross-reactive with *D. farinae* and *D. pteronyssinus* were isolated, and the MHC class II restriction specificities of these clones were mapped.[20, 21]

Proliferation assays

Polyclonal responses. PBMCs (2.5×10^5/ml) were cultured with soluble antigen in a total volume of 200 μl of complete medium in 96-well round-bottom plates. Peptides were added at various concentrations to selected wells at the initiation of cultures. After 6 days, 1 μCi of [^{3}H]TdR per well (Amersham International, Amersham, U.K.) was added to the cultures for 8 to 16 hours, and then the cells were harvested onto glass fiber filters. Proliferation as correlated with [^{3}H]TdR incorporation was measured by liquid scintillation spectroscopy. The results are expressed as mean counts per minute for triplicate cultures with SEM < 25% for all experiments with PBMCs. In those experiments in which T cell clones were used, the SEM was <10%.

Monoclonal responses. Cloned T cells (5×10^4/ml) were cultured with soluble antigen in the presence of irradiated autologous PBMCs (1.25×10^5/ml) in a total volume of 200 μl of complete medium in 96-well round-bottom plates. Peptides were added to selected wells as described for polyclonal responses. After 60 hours of incubation, [^{3}H]TdR was added to the cultures for 8 to 16 hours and then harvested as described for the polyclonal T cell proliferation assays.

Murine fibroblasts transfected with HLA-D region genes

The cloning of the DR1Dw1, DR2Dw2B5, and DRw52b genes and their cotransfection with the DRA gene into the Ltk$^-$ fibroblast cell line (DAP3) have been described elsewhere.[24-27] The transfected fibroblasts were the generous gifts of Drs. R. Lechler, J. Trowsdale, and B. Mach.

Binding assays

Murine fibroblasts transfected with the HLA-D region genes, DRw52b, DR1, and DR2Dw2B5 were examined for their capacity to bind the HA analogue, as described elsewhere.[13, 28] After incubation with peptide, containing long-chain biotin at the amino terminus, over a concentration range, the fibroblasts were then washed and stained with fluorescein avidin D (Vector Laboratories, Burlingame, Calif.). To amplify the signal a polyclonal antiavidin antibody (Vector Laboratories) was added to the cells before additional incubation with fluorescein avidin D. To control for differential expression of HLA class II molecules on the fibroblasts, cells were incubated with a fluoresceinated monomorphic anti-HLA-DR antibody (L243, Becton Dickinson, Mountain View, Calif.).[29] Stained cells were analyzed by flow cytometry with a FACS analyzer (Becton Dickinson). Only viable cells, identified by their ability to exclude propidium iodide, were analyzed.

RESULTS

Peptide inhibition of polyclonal T cell responses

Stimulation induced with D. pteronyssinus. Marked proliferation was induced in all cases when PBMCs, isolated from five atopic individuals with symptomatic perennial rhinitis, were stimulated with *D. pteronyssinus* at an optimal concentration (range, 3 to 12 μg/ml). The magnitude of the response of these individuals to HDM extracts ranged from 17 to 50 $\times$ 10^3 cpm with background responses of $<1 \times 10^3$ cpm. The addition of the HA peptide analogue, with the tyrosine at position 309 substituted by serine, to the cultures resulted in marked inhibition of the proliferative response over a concentration range (0.5 to 100 μg/ml) with maximal inhibition mediated by the HA peptide at 50 to 100 μg/ml. In the presence of the HA peptide, the percentage proliferation, as compared to the maximal HDM response achieved, varied from 10% to 34% (Fig. 1). The inhibition indicated in Fig. 1 is that mediated in the presence of maximal stimulation by HDM extract. In contrast, coculture of the keratin peptide and *D. pteronyssinus* over the same concentration range failed to decrease the observed response (Fig. 2, A). The keratin peptide was added as a control to all experiments, and in no instance was

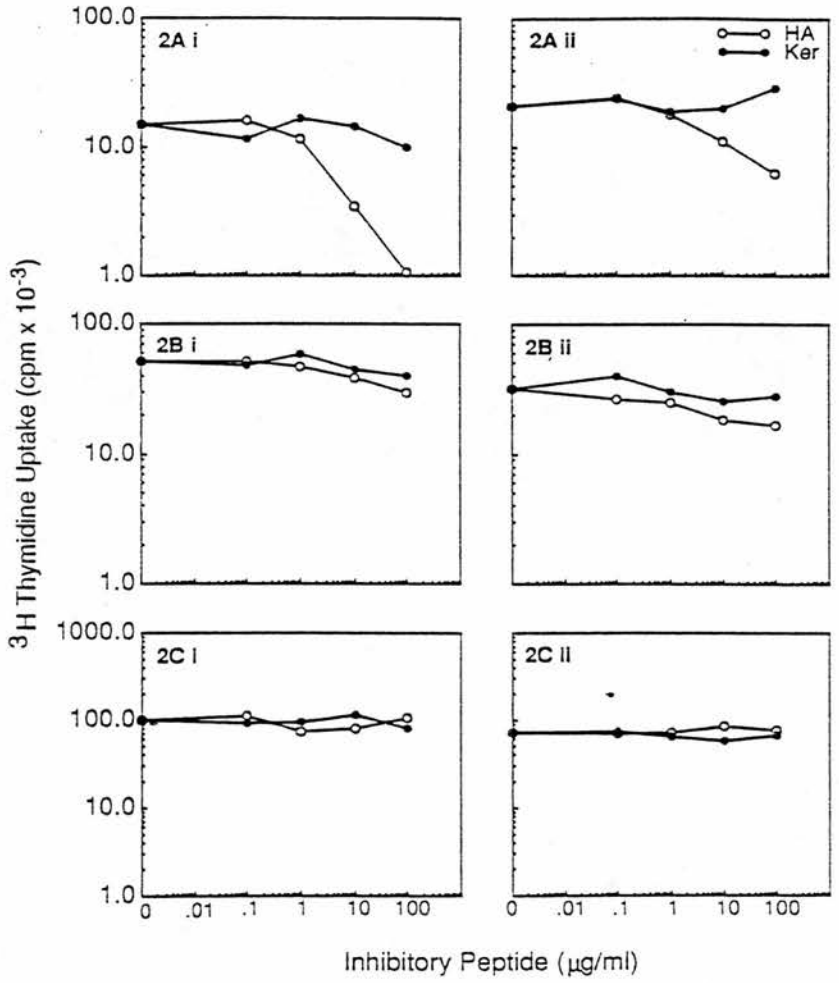

FIG. 2. Modulation of polyclonal T-cell responses. **A**, HDM. **B**, *M. tuberculosis*. **C**, PHA. PBMCs from HDM-allergic individuals, B. S. *(i)* and M. R. *(ii)*. **A**, Stimulated with antigen *D. pteronyssinus* (3 μg/ml). **B**, Stimulated with *M. tuberculosis* (3 μg/ml). **C**, Stimulated with PHA (1 μg/ml). Stimulation was in the presence of the HA S-309 (○) or keratin (●) peptides over a concentration range. Proliferation as correlated with [^{3}H]TdR incorporation was measured at day 7 and expressed as counts per minute, being the mean of triplicate cultures. The SEM was <25% in all experiments.

inhibition observed. Investigation of the kinetics revealed that preincubation with the HA peptide results in minimal enhancement of inhibition. Addition of HA 307-319, up to 24 hours after stimulation with HDM extract, is able to mediate inhibition that is not observed with the control keratin peptide (data not presented). In all cases PBMCs failed to proliferate to either of the peptides alone. Truncated analogues of the HA peptide (309-318NH_2) bind to class II molecules with the same efficiency as the native HA pep-

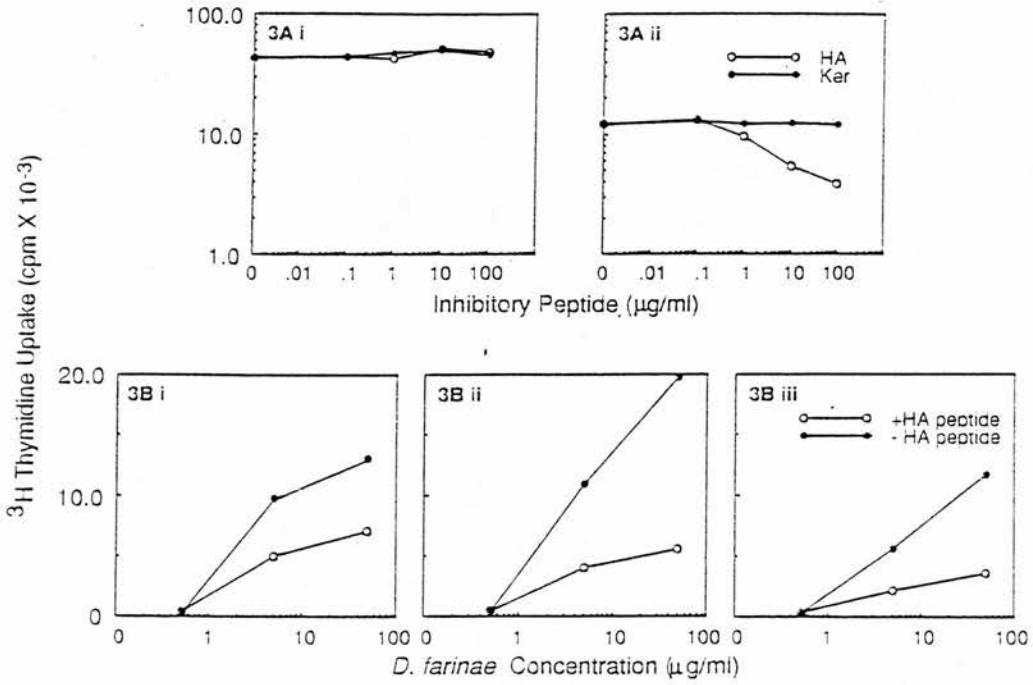

FIG. 3. Peptide modulation of monoclonal T cell responses to insolubilized anti-CD3 antibody *(i)* and HDM *(iii)*. A, Cloned T cells (DE9) were stimulated with insolubilized anti-CD3 (12 μg/ml) and IL-2 (10% vol/vol) *(i)* or *D. pteronyssinus* (3 μg/ml) *(ii)* with accessory cells in the presence of HA (○) or keratin (●) peptides over a concentration range. Proliferation was determined by [^{3}H]TdR incorporation at 72 hours. B, Inhibition of HDM-reactive T cell clones by the HA peptide. Cloned T cells DE47 (i), DE12 (ii), and DE9 (iii) were stimulated with increasing concentrations of HDM in the presence (○) or absence (●) of the HA peptide at a fixed concentration (100 μg/ml). The SEM was <10% in these experiments.

tide,[28] thus indicating that the keratin peptide, although it is shorter, provides an appropriate control to assess both biologic function and possible toxicity of residual by-products of peptide synthesis.

Stimulation induced with M. tuberculosis *soluble extract*. Polyclonal T cell proliferation observed in response to stimulation with *M. tuberculosis* soluble extract, MTSE (3 μg/ml), was only minimally inhibited by the HA analogue. The maximal inhibitory effect obtained was only 25% (B. S.) (Fig. 2, *A*) and 40% (M. R.) (Fig. 2, *A*) at 100 μg/ml, with 13% (B. S.) and 28% (M. R.) inhibition at 10 μg/ml. No inhibition of the antimycobacterial T cell response was observed in the presence of the control keratin peptide (Fig. 2, *B*).

PHA- and anti-CD3-induced activation. PHA at a concentration of 1 μg/ml induced marked proliferation in each subject (Fig. 2, *C*,*i* and *ii*). No inhibitory effect was observed with the addition of either peptide. Similarly, both peptides failed to inhibit the proliferative response of T cells stimulated directly with immobilized anti-CD3 antibody and IL-2 that mimics the recognition of peptide/MHC class II complexes (Fig. 3, *A*,*i*).

Peptide inhibition of monoclonal T cell responses

Cloned T cells (DE9: cross-reactive for both *D. pteronyssinus* and *D. farinae*, restricted by products of the B1 gene locus, DRw12, and degenerate in its restriction specificity for DR2 subtypes and DR8)[20, 21] proliferated markedly to *D. pteronyssinus* (3 μg/ml) presented by autologous irradiated PBMCs as accessory cells. The addition of the HA analogue resulted in marked inhibition of the proliferative response in a dose-dependent manner, with 60% inhi-

bition at 100 μg/ml. In contrast, coculture with the keratin peptide failed to inhibit the HDM-induced proliferation (Fig. 3, *A.ii*).

D. farinae-specific T cell clones (DE12 and DE47) restricted by the DRAB3 gene product, DRw52b,[20, 21] and T cell clone DE9 were stimulated with increasing concentrations of stimulating antigen (*D. farinae*) in the presence of autologous irradiated PBMCs as a source of antigen-presenting cells. To these cultures, the HA analogue was added at a fixed concentration (100 μg/ml), and T cell proliferation was determined. The HA peptide was able to compete with HDM for presentation to the cloned T cells and effectively inhibited the proliferation (Fig. 3, *B.i* to *iii*). However, increasing the concentration of HDM in the assays was associated with a decrease in the inhibition mediated by the HA peptide in each case. In contrast, in similar experiments, the HA analogue failed to inhibit the proliferative response of a panel of ragweed-specific T cell clones (*Amb a* reactive; DR5 restricted) stimulated by their native ligands. (Garman R, O'Hehir R, Lamb J. Unpublished results.)

Binding of the HA peptide to antigen-presenting cells expressing DRw52b HLA class II molecules

To examine the binding of the HA peptide to DRw52b in isolation from other HLA class II molecules, the appropriate genes were transfected in murine fibroblasts. Murine fibroblasts transfected with the DRw52b gene were able to bind the HA peptide in a dose-dependent manner in contrast to the control fibroblasts expressing DR1 or the untransfected cell line (DAP3), as determined by the level of fluorescence (Fig. 4). Similarly, the HA peptide was able to bind in a dose-dependent manner to murine fibroblasts transfected with DR2Dw2B5 genes, which were functionally able to present HDM to clone DE9.[21]

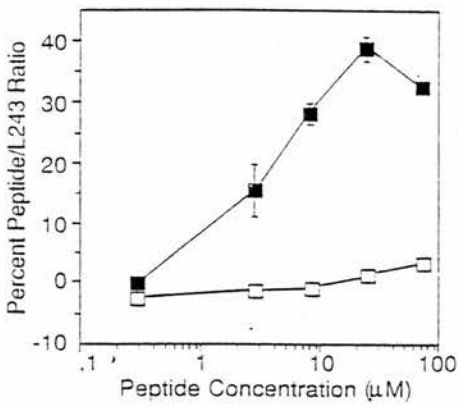

FIG. 4. Binding of the HA peptide to DRw52b HLA class II molecules expressed on the surface of murine fibroblasts. Murine fibroblasts expressing DRw52b (■) or DR1 (□) were incubated with biotinylated peptide, stained, and analyzed by flow cytometry. Backgrounds as determined by incubation of the peptide with untransfected fibroblasts were subtracted. Results are expressed as the percentage of peptide bound relative to the binding of the anti-HLA-DR antibody (L243) to normalize for differential HLA class II expression.

DISCUSSION

The realization that CD4+ T cells play a critical role in allergic responses allows the reassessment and development of novel therapeutic approaches. The practice of hyposensitization has been widely used, but the results have been variable[30-32] and the immunologic mechanisms remain ill defined. Potentially, the capacity of nonallergen components to compete with allergenic peptides themselves for occupancy of the antigen-combining site of MHC class II molecules could prevent the activation of allergen-specific T cells and the subsequent induction of IgE synthesis. The observation that a nonstimulatory peptide analogue of influenza HA (residues 307-319, with the tyrosine at position 309 substituted by serine) could bind strongly to murine fibroblasts expressing the DRAB3 gene product, DRw52b, prompted us to investigate the potential of this peptide to inhibit recognition of specific antigen by immune T cells isolated from the peripheral blood of symptomatic HDM-allergic individuals. For the management of specific allergic responses by manipulation of the immune system, consideration of antigen and HLA-restriction specificity of T cell recognition is necessary. In contrast to the serologic response that is predominantly cross-reactive,[33, 34] T cell recognition of dust mite is also directed toward components of either of the two main species of HDM (*D. pteronyssinus* and *D. farinae*).[22] The HA peptide inhibits both polyclonal T cell responses to HDM and that of individual T cell clones of different specificities, suggesting that the competing peptide prevents activation of most dust mite-reactive T cells. However, the observation of only partial inhibition of the antimycobacterial and antiragweed T cell responses demonstrates that there is a certain degree of specificity and that overall T cell responsiveness is not ablated. The HDM and mycobacterial T cell responses could be restricted by the same MHC class II molecules but with most mycobacterial epitopes binding at higher affinity, such that the S-309 peptide was unable to compete. Alternatively, only a minor component of the T cell responses to HDM and mycobacteria might have overlapping restriction specifici-

ties. The failure to block either the PHA or anti-CD3 antibody-induced proliferation, acting directly on T cells via CD2 and T cell-specific antigen receptor, respectively, suggests that antigen competition for MHC class II binding is the mechanism involved.

The apparently high concentration of the HA peptide required to inhibit HDM-induced proliferation may be a function of antigen processing in that the peptide fails to associate with the class II molecules as efficiently as the HDM extract. As a result the peptide may be competing for occupancy of the antigen-combining site of the appropriate class II molecules expressed only on the cell membrane and not in the intracellular compartment of the antigen-presenting cells. However, caution must be used in extrapolating from these in vitro studies on the potential efficacy of peptide inhibition in vivo.

Nevertheless, peptide engineering and examination of different routes of drug delivery may enhance the immunomodulatory effects. Additionally, the ability to inhibit the proliferative response of T cell clones indicates that the induction of additional regulatory T cells[32, 35] does not need to be a prerequisite for hyposensitization. Although the administration of components of HDM may appear to be the most appropriate means of achieving dust mite hyposensitization,[31] the use of nonstimulatory peptides, such as the derivative of the naturally occurring influenza HA, as we have described here, minimizes the risk of triggering an allergic response and therefore may prove to be the preferable therapeutic approach.

Examination of the genetic restriction of polyclonal and monoclonal T cell responses to HDM has revealed that products of HLA-DRAB3 class II genes (DRw52) function as a dominant restriction element. The DRw52 class II molecules, which are associated with the HLA-DR 3, 5, 6, and 8 alleles, may contribute to the high frequency of dust mite allergy. However, the polygenic nature of allergic responses[36] suggests that specific responsiveness to HDM may not be regulated by the DRB3 gene products alone but involves other genes, such as the "atopy gene" recently mapped to chromosome 11q.[37] Interestingly, the HA peptide was able to bind to DRw52b expressed on the surface of accessory cells and to inhibit the antigen-dependent response of cloned HDM-specific T cells with that restriction specificity. A minor component of the T cell repertoire reactive with HDM appears to be restricted by DRAB1 gene products and, in some cases, this has been mapped to the locus of DRw12.[20, 21] Clone DE9 is restricted by DRw12 and degenerate on accessory cells expressing DR2Dw2B5, DR2Dw21B1, and DR8. These HLA-DR molecules have identity at positions 70 and 71 in the third variable region of the β-1 domain that forms the α-helical wall of the antigen-combining site of class II histocompatibility antigens.[38] This suggests that residues 70 and 71 are able to regulate T cell antigen recognition independent of those polymorphic residues forming the floor of the antigen-combining site.[39] Binding studies with murine fibroblasts expressing DR2Dw2B5 molecules demonstrated that the HA analogue was able to bind to this HLA-DR molecule and accounted for the ability of the peptide to compete for recognition of HDM by this clone of T cells. Additional binding studies on a panel of Epstein-Barr virus–transformed B cell lines have compared binding of the native HA peptide (tyrosine at 309) and the monosubstituted analogue with serine at position 309. The results of these experiments demonstrated a very restricted binding of the S-309 HA analogue as compared with the highly permissive binding of the native HA peptide (Y-309).[28] Interestingly, most B cell lines that bound HA S-309 expressed both B1 and B3 gene products. Further analysis with murine fibroblasts confirmed the high binding to DRw52b and DR2Dw2B5, consistent with the ability of the peptide to compete with HDM recognition in the polyclonal and monoclonal T cell responses.

As precedent for the use of a nonstimulatory peptide to regulate immune responsiveness, Adorini et al.[18] demonstrated that a self-peptide derived from murine lysozyme, and differing by only three amino acid residues from hen egg white lysozyme, was able to inhibit presentation of hen egg white lysozyme in vivo in high-responder strains. Competitive inhibition with "nondisease-related" peptides has also been demonstrated to prevent the development of experimental allergic encephalomyelitis.[40] An approach such as this may be of clinical benefit in the treatment of localized allergic disease, such as perennial rhinitis. The immune responsiveness of atopic individuals to allergen is clearly an aberrant response, and a therapeutic regimen that down regulated the specific reactivity would be beneficial.

We thank Dr. B. Mach for helpful scientific discussions.

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Brief communications

T-cell receptor peptides that inhibit the T-cell response to allergen induce transforming growth factor-β_1 production

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Recent findings indicate restricted usage of T-cell receptor (TcR) β chain variable region 3 (Vβ3) gene elements in the human polyclonal T-cell response to the allergens of house dust mite (HDM).[1, 2] Furthermore, we have reported that a peptide analog of TcR-Vβ3 CDR2 specifically inhibits the polyclonal proliferative response of human Vβ3−T cells to unfractionated *Dermatophagoides pteronyssinus* extract.[2] Although the mechanism of inhibition remains to be defined, there is increasing evidence that immunosuppressive cytokines may play an important role in the downregulation of immune responsiveness in a number of experimental systems.[3-5] In this study we reveal that the Vβ3-CDR2 peptide induces the production of the inhibitory cytokine transforming growth factor-β_1 (TGF-β_1) but that no IL-4, IL-10, or interferon-γ (IFN-γ) activity was detectable in the same culture supernatants.

Abbreviations used

HDM:	House dust mite
IFN-γ:	Interferon-γ
MTSE:	*Mycobacterium tuberculosis* soluble extract
PBMCs:	Peripheral blood mononuclear cells
TcR:	T-cell receptor
TGF-β_1:	Transforming growth factor-β_1
Vβ:	Variable region of the β chain of the T-cell receptor

METHODS

Separation of peripheral blood mononuclear cells (PBMCs) from healthy adults by Ficoll-Hypaque density centrifugation and $CD4^-$ and $CD8^-$ T-cell depletion protocols (routinely <3% contamination) has recently been described.[2] PBMCs (5×10^6) were cultured in 1 ml of supplemented RPMI-1640 media[2] (Gibco, Paisley, Scotland) and 5% AB^- human serum with 10 μg/ml *Mycobacterium tuberculosis* soluble extract (MTSE),[2] 25 μg/ml HDM extract,[2] 25 to 100 μg/ml Vβ3-CDR2 peptide (GLGLRLIYFSYDVKMKEKGDI), Vβ6.7a-CDR2 peptide (SPLGSKDPASNG), murine Vβ8-CDR2 peptide (IDGKETSDAVYSYHIL),[2] or 10 μg/ml phytohemagglutinin plus 10 ng/ml phorbol myristate (PHA/PMA; Sigma Chemical Co., St. Louis, Mo.). Supernatants from these cultures were harvested after 5 days and frozen at −20° C until they were tested for cytokine activity as described below.

IL-4 and IFN-γ activity were measured with ELISAs (Eurogenetics, Teddington, U.K.). IL-10 was measured by ELISA with antibodies kindly provided by Dr. Anne O'Garra (DNAX, Palo Alto, Calif.). The Mv1Lu bioassay was used to measure TGF-β_1 activity.[6] Latent TGF-β_1 was converted to the active form by reducing the pH to less than 3.0 for 30 minutes at room temperature. TGF-β_1 activity was inversely proportional to Mv1Lu cell growth, as determined by tritiated thymidine incorporation,[2] when tests were performed in replicates of 3 to 6 wells, with standard deviations routinely less than 20%.

RESULTS

Supernatants harvested from PBMCs cultured with 25 to 100 μg/ml Vβ3-CDR2 peptide were tested in the Mv1Lu growth inhibition assay for TGF-β_1 bioactivity. The Vβ3-CDR2 peptide induced a dose-dependent production of active

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Supported by grants from the Wellcome Trust. L.G. was funded by the Arthritis and Rheumatism Council of Great Britain and R.E. O'H is a Wellcome Senior Clinical Fellow.

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J ALLERGY CLIN IMMUNOL 1996;97:707-9.

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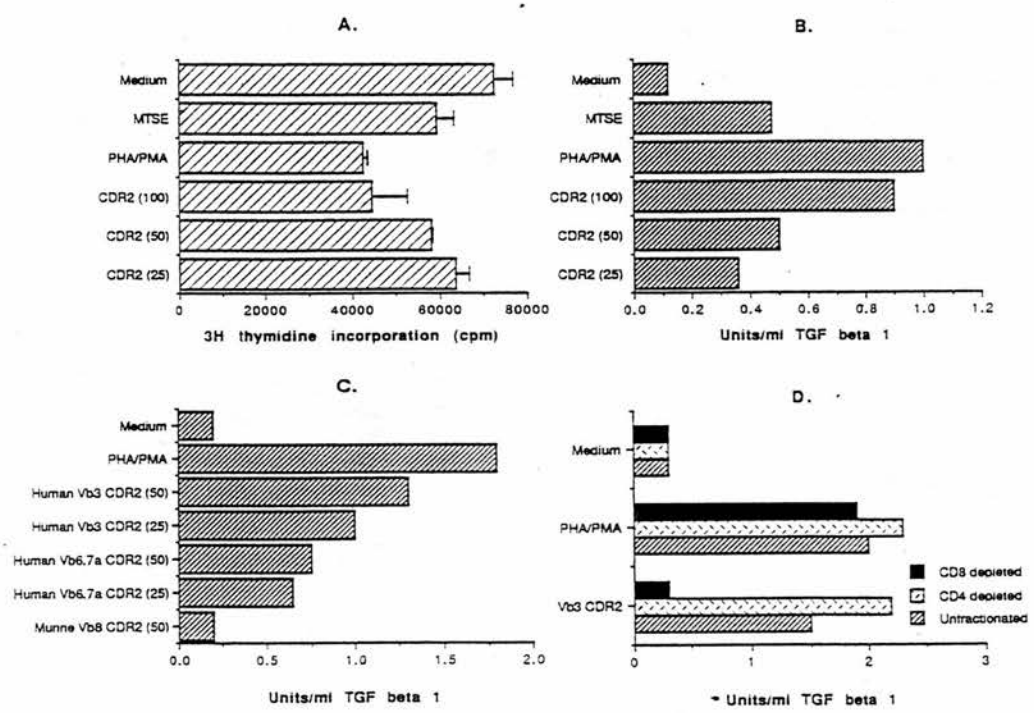

FIG. 1. Induction of TGF-β_1 activity by TcR peptides. Unfractionated human PBMCs (**A-D**) or CD4- and CD8-depleted PBMCs (**D**) were cultured at 5×10^6 cells/ml in medium, with 10 μg/ml PHA plus 10 ng/ml PMA, 10 μg/ml MTSE, or the indicated concentrations (25 to 100 μg/ml; 50 μg/ml in **D**) of TcR peptides for 5 days. Culture supernatants were then harvested and assayed for TGF-β_1 bioactivity as described in Methods. Data are presented in **A** as mean counts per minute ± standard deviation of replicate cultures and **B, C,** and **D** as units per milliliter of TGF-β_1 activity as extrapolated from a standard curve obtained with recombinant human TGF-β_1. A and B represent data from the same experiment.

TGF-β_1, with levels comparable to those induced by PHA plus PMA, a potent stimulus of TGF-β_1 production, but more than those induced by antigen (Fig. 1, *A* and *B*). Comparable results were observed in all nine individuals tested (mean ± SD): TGF-β_1 in medium, 0.31 ± 0.24 U/ml; PHA/PMA, 1.52 ± 0.85 U/ml; TcR-Vβ3 CDR2 peptide, 1.43 ± 0.79 U/ml; and HDM, 0.585 ± 0.53 U/ml. A second CDR2 peptide derived from the human Vβ6.7a gene element induced lower but detectable levels of TGF-β_1 in two individuals tested (Fig. 1, *C*, and data not shown). This was particularly interesting because Vβ6.7a-TcR usage was detected, albeit at a lower level than TcR-Vβ3, in a panel of HDM-specific T-cell clones.[1] A control murine CDR2 peptide, derived from murine TcR-Vβ8, failed to stimulate TGF-β_1 production (Fig. 1, *C*).

To demonstrate that the TGF-β_1 bioactivity detected in our cultures was directly synthesized after peptide stimulation of PBMCs and not caused by serum contaminants, cells were cultured for 24 hours with various stimuli in serum containing medium, washed extensively, and then recultured in the absence of serum for an additional 3 days. Serum-free supernatants were harvested and assayed for active versus total (latent plus active) TGF-β_1 content after in vitro conversion of latent TGF-β_1 by acid treatment. Supernatants from PBMCs cultured in medium alone or with Vβ3-CDR2 peptide contained less than 0.3 and 2.4 active total U/ml TGF-β_1 compared with 1.4 and 13 U/ml TGF-β_1, respectively.

Cell depletion studies previously suggested that $CD8^-$ T cells play a central role in mediating CDR2 peptide inhibition of the T-cell response to

HDM allergens.[2] The induction of active TGF-β_1 in unfractionated, CD4-depleted, and CD8-depleted PBMCs was therefore compared. As shown in Fig. 1, *D*, a representative experiment of four performed, high levels of TGF-β_1 bioactivity were detectable in all three groups after PHA/PMA stimulation. In contrast, Vβ3-CDR2 peptide induced TGF-β_1 production in unfractionated and CD4-depleted, but not in CD8-depleted, PBMC cultures, suggesting that the $CD8^-$ T-cell fraction is the major cell type responding to this TcR peptide. When IL-10 levels were measured in the same supernatants, only PHA/PMA stimulation resulted in marked cytokine production, as measured by ELISA. Furthermore, induction of IL-10 by PHA/PMA was readily detected in unfractionated and CD8-depleted (6.7 and 4.1 U/ml respectively), but not CD4-depleted cultures (0.6 U/ml; medium control, HDM, or CDR2 peptide for all groups <1.0 U/ml).

The Vβ3-CDR2 peptide preparation also failed to induce IFN-γ (n = 6; medium, 3.8 ± 4.5; PHA/PMA, 329 ± 47.8; CDR2, 2.8 ± 2.8; HDM, 8.2 ± 9.5; MTSE [n = 4], 247.5 ± 129 pg/ml) or IL-4 (medium, HDM, or CDR2 peptide <6 pg/ml; PHA/PMA, 40 pg/ml) production as measured by specific ELISA. IL-2 activity, measured in the CTLL growth assay, was also not detected in 24-hour culture supernatants of cells cultured in medium or peptide, but was found in the control HDM and PHA/PMA supernatants (data not shown).

DISCUSSION

In this report we demonstrate that peptides derived from the human TcR-Vβ3 CDR2 region induce production of active TGF-β, by PBMCs in culture. IL-10, IFN-γ, IL-4, or IL-2 activity in the same cultures was below the limits of detection. Strong induction of all cytokines was observed after stimulation with PHA/PMA. Furthermore, depletion of $CD8^-$ T cells resulted in loss of TGF-β_1 production, suggesting that $CD8^-$ T cells were responsible for TGF-β_1 production after TcR peptide stimulation. Similarly, inhibition of the proliferative response of human PBMCs to HDM allergens by Vβ3-CDR2 peptide required the presence of $CD8^-$ T cells. Our findings suggest that this inhibition may, in part, be mediated through the induction of immunosuppressive cytokines, such as TGF-β_1. Nevertheless, neutralizing anti-TGF-β_1 antibody failed to reverse CDR2 peptide–mediated inhibition of the HDM response. This observation is not surprising, given the likely requirement for close interaction of two different cell types, in this case the putative $CD8^-$ regulatory T cells with $CD4^-$, TcR-Vβ3$^-$ HDM-reactive cells, for the conversion of latent to active TGF-β_1 activity as proposed in other experimental systems.[7] If this is so, it is unlikely that "free" cytokine would be available for antibody neutralization.

TGF-β_1 acts directly on T cells by inhibiting IL-1 and IL-2 receptor expression and cytokine production, as well as indirectly by inhibiting T-cell activation by modulation of antigen-presenting cell function.[3] In addition, TGF-β_1 inhibits the switch to germline ε transcription and IgE production, while selectively inducing a switch to α chain and IgA transcription. A number of inhibitory mediators induce production of TGF-β_1, including cyclosporine,[5] the synthetic glucocorticoid dexamethasone,[4] and, most recently in this report, TcR peptides. These observations have led to growing recognition of an important role for this immunosuppressive cytokine in the modulation of allergic and other immune phenomena.

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2.3 ANTIGEN MEDIATED MODULATION OF T CELL FUNCTION

2.3.2. ALTERED T CELL RECEPTOR LIGANDS

Tsitoura DC et al: *J Immunol* 1996, **157**:2160-2165.

Tsitoura DC et al: *Immunology* 1997, **92**:10-19.

Altered T Cell Ligands Derived from a Major House Dust Mite Allergen Enhance IFN-γ but Not IL-4 Production by Human CD4$^+$ T Cells[1]

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Changes in the affinity of the interaction between T cell Ag receptors and their ligands can modulate selected T cell effector functions. Since both allergen-specific Th2 and Th0 cells are present in the peripheral CD4$^+$ T cell pool of atopic individuals, the potential to inhibit cytokine production by Th2 cells and promote Th1-type cytokines from Th0 cells may contribute to the down-regulation of allergic inflammation. The aim of this study was to investigate the effects of peptide analogues of a dominant T cell epitope of the group II allergen derived from house dust mite, residues 28 to 40, on proliferation and cytokine production by human Th2 and Th0 cells. From both functional competition and proliferation assays, using analogues substituted with alanine or charged amino acids, the influence of different positions in p28–40 on TCR recognition and/or MHC class II binding was determined. For the specific Th0 cells, generally those analogues that lead to a reduction in proliferation also decreased both IL-4 and IFN-γ production. However, the p28–40 analogues with alanine residues at positions 34 and 36 altered the IFN-γ:IL-4 ratio by selectively enhancing IFN-γ secretion. In the case of Th2 cells, stimulation with the peptide analogues induced different patterns of effector function. Selected analogues were capable of inducing IL-4 production in the absence of proliferation, whereas in response to other peptide variants of p28–40, both IL-4 production and proliferation were inhibited. *The Journal of Immunology,* **1996, 157: 2160–2165.**

The qualitative nature of allergen-specific immune responses in sensitized individuals, based on the analysis of peripheral T cells (1–3) and clinical biopsies (4), suggests that both Th2 and Th0 cells contribute to the functional phenotype of the peripheral CD4$^+$ T cell repertoire (5). The functional activity of these T cell subsets is characterized by their cytokine response profiles, with Th2 cells producing IL-4, IL-5, IL-10, and IL-13, and Th0 cells synthesizing IL-2, IFN-γ, and TGF-β in addition to Th2 cytokines (6–9). The induction of allergen-specific IgE requires the presence of Th2-type cytokines, and it is in part the balance between the agonist and antagonist effects of IL-4 and IFN-γ that regulates IgE synthesis (10). Therefore, the potential to inhibit IL-4 production and/or promote IFN-γ secretion may contribute in the long term to the down-regulation of IgE and be of clinical benefit to individuals with atopic allergy.

In the presence of costimulatory signals, recognition of peptide fragments of allergen bound to MHC class II molecules by TCR is required for the activation of all CD4$^+$ T cell subsets (11, 12). However, there is evidence indicating that altering the affinity of the interactions between TCRs and their cognate ligands can lead to the selective activation of T cell effector functions (13–18). This is illustrated by the finding that murine Th1 cells, when stimulated with an altered T cell ligand, failed to produce cytokines or proliferate, yet retained cytolytic activity and up-regulated their IL-2 receptors (13). Furthermore, stimulation of murine Th2 cells with analogues of an immunogenic peptide in which a secondary TCR contact residue had been altered by conservative amino acid substitutions was sufficient to induce IL-4 production and provide B cell help, but not clonal expansion (14). Peptide analogues with partial agonist activity have been reported that are capable, in the present of live APC, of inducing anergy in both Th1 and Th2 cells. These cells fail to proliferate when restimulated with their natural ligand, although in the case of Th2 cells, their ability to produce IL-4 is retained (15, 16). Moreover, observations made in vivo in mice revealed that structural differences in antigenic peptides or class II molecules of different MHC haplotypes can influence the development of Th1 or Th2 responses (17). In particular, it appeared that high affinity MHC/peptide interactions with the TCR favored the induction of Th1 responses, while interactions of low affinity resulted in the activation of Th2 cells. In a recent report it has been demonstrated that stimulation of T cells in vitro with an altered peptide ligand derived from the myelin proteolipid protein induces Th2 and Th0 cells, which, when adoptively transferred to mice, confer protection from experimental autoimmune encephalomyelitis (19).

The aim of the experiments reported here was to determine whether peptide analogues of a major human T cell epitope of the group II allergen of house dust mite (HDM)[3] *Der p* II (20), residues 28 to 40, with single amino substitutions introduced at each position can modulate T cell proliferation and cytokine production

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Received for publication April 8, 1996. Accepted for publication June 18, 1996.

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[1] This work was supported by grants from the Wellcome Trust and the Medical Research Council, U.K.

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[3] Abbreviations used in this paper: HDM, house dust mite; *Der p* II, the group II allergen of *Dermatophagoides pteronyssinus*; ^{3}HIdThd, tritiated thymidine.

0022-1767/96/$02.00

Table I. *Amino acid sequence of wild type and analogues of p28–40*

28												40
I	I	H	R	G	K	P	F	Q	L	E	A	V
A												
	A											
		A										
		E	A									
		K	E	A								
			K	E	A							
				K	E	A						
					R	K	A					
							K	A				
									A			
										A		
											A	
												A

by *Der p* II-specific Th2 and Th0 cells isolated from an atopic individual.

Materials and Methods

Antigens

The *Der p* II peptides, residues 81 to 96 (VKGQQYDIKYTWNVPK) and 28 to 40 (IIHRGKPFQLEAV), and analogues of p28–40 were synthesized on a Multipeptide Synthesis block BT7400 (Cambridge Research Biochemicals, Northwich, U.K.) using 4-(2',4'-dimethoxyphenyl F-moc aminomethyl)phenoxy resins (Novasyn Chemicals Ltd., Nottingham, U.K.) as previously described (21). Couplings were performed using F-moc side chain protected pentafluorophenyl or oxo-benzotetazine amino acid esters. Full-length peptides were cleaved from the resin and deblocked by treatment with TFA/thioanisole/ethanedithiol (94/5/2.5, v/v/v) for 1 h and then precipitated and washed twice with ether. The precipitates were dissolved in 10% formic acid (v/v) and freeze-dried for 48 h. Each peptide produced a single peak by reverse phase HPLC. The peptide analogues used in this study are listed in Table I.

*Isolation of Der p II-reactive, DRB1*1101-restricted, cloned human Th0 and Th2 cells*

Cloned T cells of AC1 (Th0) and AC2 (Th2) were isolated from a HDM atopic individual (DRB1*0701, DRB1*1101; DQ2, DQ7 (3); DPB1*0101/0402) by limiting dilution cloning from a long term T cell line as described previously (20). The T cell clones were maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 IU/ml penicillin/streptomycin (Life Technologies, Paisley, Scotland), and 5% inactivated human A^- serum (National Blood Transfusion Service, Edgware, U.K.) by stimulation with the p28–40 peptide at optimal concentrations and irradiated histocompatible PBMC (5×10^5/ml; 3000 rad) as a source of APCs together with IL-2 (10%, v/v; Lymphocult T, Biotest Folex, Frankfurt, Germany). In all experiments, T cell clones were rested for 7 to 8 days after the last addition of Ag and APCs.

T cell proliferation assays

Cloned T cells (2×10^4/well) were cultured in flat-bottom 96-well microtiter trays (Nunc, Life Technologies) with native peptide or analogues of p28–40 (3 and 30 μg/ml) in the presence of APCs. Mitomycin C-treated murine fibroblasts expressing HLA-DRB1*1101 (2×10^4 cells/well) were used for Ag presentation. Cultures were pulsed with tritiated thymidine (1 μCi/well; [^{3}H]dThd; Amersham International, Aylesbury, U.K.) after 48 h and harvested 8 to 16 h later. Proliferation was determined by [^{3}H]dThd incorporation by liquid scintillation spectroscopy. The results are expressed as mean counts per minute for triplicate cultures, with an SEM < 20%.

Cellular competition assays

The binding of native p28–40 peptide and analogues to DRB1*1101 was assessed by their capacity to inhibit the proliferation of a DRB1*1101-restricted T cell clone (AC29) reactive with p85–97 (20). Peptide analogues of p28–40 (50 μg/ml) together with the peptide p81–96 (5 μg/ml) were added to mitomycin C-treated murine fibroblasts expressing DRB1*1101. To these cultures T cells of clone AC29 were added, and proliferation was measured by [^{3}H]dThd incorporation. AC29 cells were isolated and expanded as described above. The results are expressed as the percentage of inhibition of AC29 proliferation relative to that induced by the native p28–40 peptide.

Measurement of IFN-γ and IL-4

Supernatants were collected at 48 h from T cells stimulated with peptide or cultured in medium alone. Triplicate wells were pooled, and IFN-γ and IL-4 were measured by ELISA kits as described previously (22).

Results

Effect of stimulation with p28–40 peptide analogues on Ag recognition by specific T cell clones representative of the Th0 and Th2 functional phenotypes

The T cell clones AC1 and AC2, both of which are reactive with residues 28–40 of *Der p* II and restricted by HLA-DRB1*1101, exhibit the cytokine profiles of Th0 and Th2 cells, respectively (20, 23). Initially, AC1 and AC2 T cells were stimulated with peptide analogues of p28–40 synthesized with alanine substituted sequentially at each position (Table I), and the proliferative responses were determined (Fig. 1, *A* and *B*). The antigenicity of the analogues with substitutions at positions 31, 32, and 33 was markedly reduced compared with that of the native peptide for both T cell clones, although minor differences in the pattern of proliferation in response to these analogues were observed. For example, the response of AC1 T cells was more susceptible to the alanine substitution at position 31, whereas A^{32} had a greater inhibitory effect on T cell recognition by AC2 T cells. The patterns of responsiveness and effector function were not differentially affected by variations in the concentrations of peptides (data not shown). The presence at alanine at other positions in p28–40 had minimal or no effect on the proliferative responses of either of the T cell clones.

To further examine the relative importance of different residues in p28–40 on T cell recognition, in particular those at positions 31, 32, and 33, peptides with charged amino acids were synthesized (24). Arginine (R) occurs at position 31 in the native peptide, but when replaced with either a conservative (K^{31}) or a radical (E^{31}) substitution, a reduction in the proliferative response of both AC1 (Fig. 1*C*) and AC2 (Fig. 1*D*) was observed. Similarly, both T cell clones responded weakly to the peptide analogue in which glycine at position 32 had been replaced with lysine (K^{32}). In contrast, although AC2 (Fig. 1*D*) failed to recognize the analogue E^{32}, the response of AC1 was only partially inhibited (Fig. 1*C*). The conservative substitution of R^{33} for K^{33} induced proliferation similar to that of the native peptide, whereas E^{33} was only weakly antigenic for both T cell clones. With the exception of E^{30}, the introduction of charged residues at positions 30, 34, and 35 markedly reduced T cell proliferation.

The modulatory effect of the analogues on T cell recognition may occur as the result of altering either the binding of the peptide to HLA-DRB1*1101 or the affinity of the peptide-TCR interaction. To test the first of these possibilities, native peptide and analogues of p28–40 were investigated for the ability to competitively inhibit presentation of p81–96 to the T cell clone AC29 (Fig. 2). If the binding capacity of a *Der p* II 28–40 analogue to DRB1*1101 is not affected by the introduced substitution, it would be expected to equally inhibit proliferation of AC29 cells to the native 28–40 peptide. Indeed, this was the case with analogues substituted at positions 28, 31, 32, 34, 36, 37, and 40. In contrast, A^{29}, E^{33}, and A^{38} were more efficient at inhibiting the proliferation of AC29 cells, suggesting that they bind with higher affinity to DRB1*1101 than the native peptide. However, since A^{30}, R^{33}, and A^{35} were less effective than native peptide at inhibiting AC29 proliferation, it is likely that they bind with lower affinity to DRB1*1101. From the combined results of proliferation and functional competition

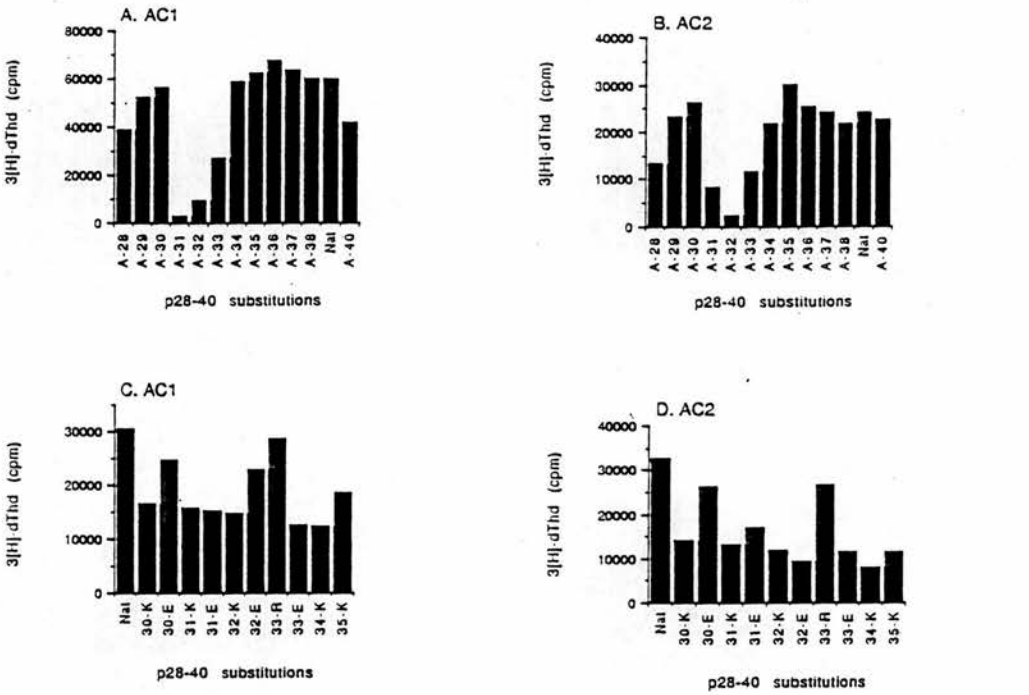

FIGURE 1. The proliferative response of *Der p* II (p28–40)-specific Th0 and Th2 cells to peptide analogues of p28–40. T cells of clone AC1 (Th0) and AC2 (Th2) were stimulated with p28–40 peptide analogues in the presence of mitomycin C-treated murine fibroblasts expressing HLA-DRB1*1101 as a source of APCs. Proliferation was determined by [^{3}H]dThd incorporation. Background responses of T cells cultured with APCs in the absence of Ag were <450 cpm.

assays, it appears that amino acids at positions 31 and 32 are critical for T cell recognition by both AC1 and AC2 T cells. In contrast, due to the different capacities of A^{33}, E^{33}, and R^{33} to competitively inhibit AC29 T cell proliferation, position 33 appears to affect T cell recognition as well as binding to MHC class II molecules.

Effect of p28–40 peptide analogues on cytokine production by the T cell clones AC1 (Th0) and AC2 (Th2)

The p28–40 analogues were investigated for their ability to modulate cytokine production by AC1 (Th0) and AC2 (Th2) cells. In response to stimulation by the p28–40 analogues with alanine at positions 28, 31, 32, 33, and 40, the production of IL-4 by AC1 cells was markedly reduced compared with that elicited by the native peptide (Fig. 3*A*). Stimulation with A^{29} and A^{30} also resulted in diminished levels of IL-4 secretion. Although the analogues A^{31}, A^{32}, and A^{33} markedly down-regulated the proliferative response of the Th2 cells (AC2), their inhibitory effects on IL-4 secretion were minimal (Fig. 3*B*), demonstrating that IL-4 production and proliferation could be dissociated. Background release of IL-4 by both AC1 and AC2 cells in the absence of peptide was <10 pg/ml. The additional analogues with charged amino acid substitutions were also tested for their ability to stimulate IL-4 production by AC1 and AC2 cells. Compared with the native peptide glutamic acid or lysine at positions 31, 32, 34, and 35 as well as E^{33} reduced the capacity of AC1 cells to produce IL-4 by 35%

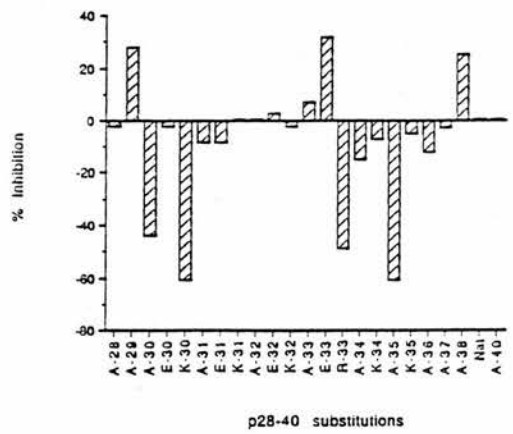

FIGURE 2. Assessment of the binding of p28–40 peptide analogues to HLA-DRB1*1101. Peptide analogues of p28–40 (50 μg/ml) were added with the stimulating peptide p81–96 (5 μg/ml) to mitomycin C-treated murine fibroblasts expressing DRB1*1101 (2×10^4/well). To these and control cultures containing no p28–40-derived peptides, AC29 T cells (p82–96 specific and DRB1*1101 restricted) were added (2×10^4/well), and proliferation was determined by [^{3}H]dThd incorporation. The results are expressed as the percentage of inhibition relative to that induced by the native p28–40 peptide.

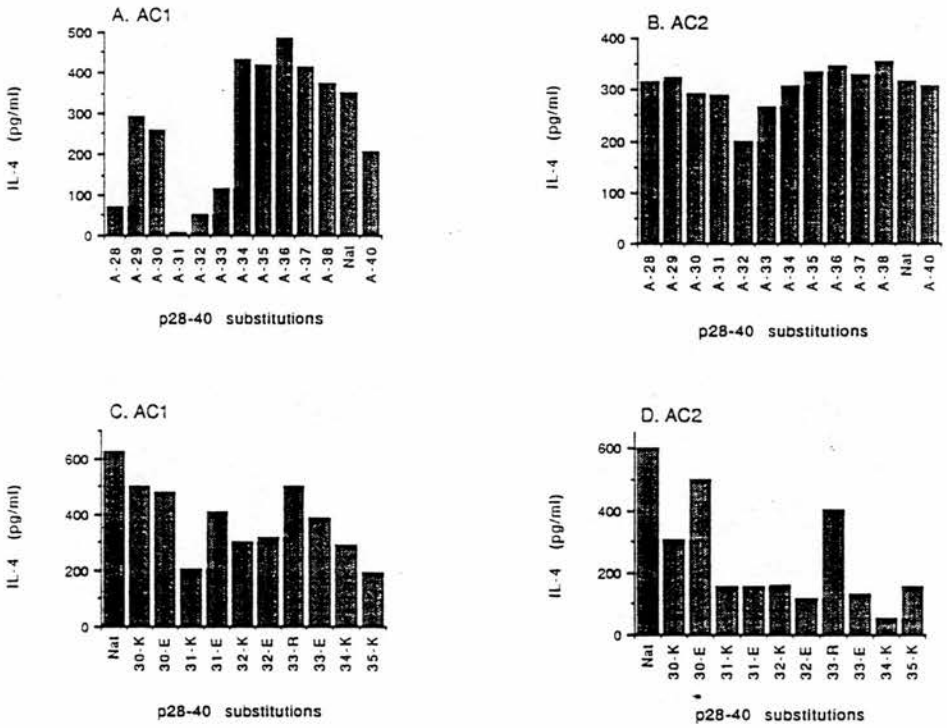

FIGURE 3. The effects of peptide analogues on IL-4 production by *Der p* II (p28–40)-specific Th0 and Th2 cells. T cells of clone AC1 (Th0) and AC2 (Th2) were stimulated with p28–40 peptide analogues in the presence of mitomycin C-treated murine fibroblasts expressing HLA-DRB1*1101 as a source of APCs. Supernatants were collected at 48 h, and the levels of IL-4 were determined by ELISA.

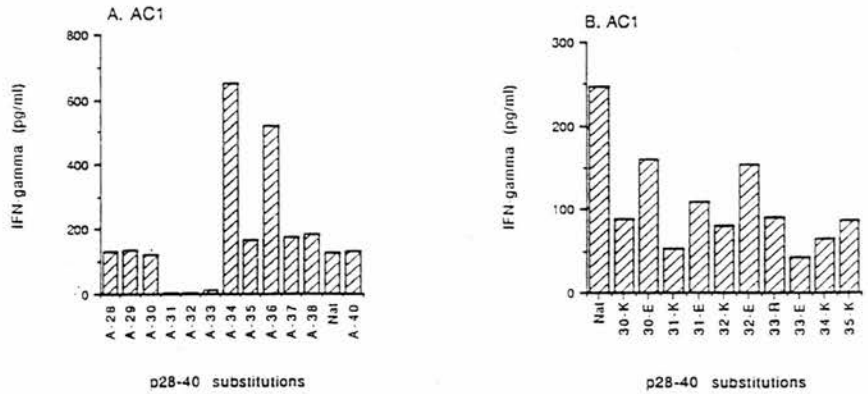

FIGURE 4. The effects of peptide analogues on IFN-γ production by *Der p* II (p28–40)-specific Th0 cells. T cells of clone AC1 (Th0) were stimulated with p28–40 peptide analogues in the presence of mitomycin C-treated murine fibroblasts expressing HLA-DRB1*1101 as a source of APCs. Supernatants were collected at 48 h, and the levels of IFN-γ were determined by ELISA.

or more (Fig. 3*C*). The analogues E^{30} and K^{30} induced IL-4 production by both T cell clones, although for Th2 cells, E^{30} was the most potent of these two peptides (Fig. 3, *C* and *D*). Analogues with charged amino acids at positions 31, 32, and 33 reduced IL-4 production by AC2 cells, although in the case of R^{33}, IL-4 secretion was diminished by only 33% (Fig. 3*D*).

The ability of p28–40 peptide analogues to stimulate IFN-γ production by Th0 cells (AC1) was also investigated. The analogues A^{31}, A^{32}, and A^{33} all failed to induce IFN-γ production (Fig. 4*A*), and similarly, stimulation by peptides with charged residues at these positions was also unable to elicit the release of IFN-γ. Substitutions with alanine at positions 28, 29, 30, 35, 37,

38, and 40 induced the same level of IFN-γ secretion as the native peptide (Fig. 4*A*). In contrast, A^{34} and A^{36} increased IFN-γ secretion compared with native p28–40, with enhancements of five- and fourfold, respectively (Fig. 4*A*). None of the analogues with charged residue substitutions was as potent as the native peptide at inducing IFN-γ secretion (Fig. 4*B*); however, the IFN-γ response profile of AC1 cells was similar to that observed for IL-4 in response to stimulation with these analogues. Undetectable levels of IFN-γ were produced by AC1 cells cultured with APCs in the absence of peptide.

Discussion

The potential to modulate the qualitative nature of immune responses with structural analogues of antigenic peptides was investigated in vitro for T cell responses to a major human T cell epitope of the HDM. In atopic disorders it may be possible to achieve clinical improvement by decreasing the ratio of Th2 to Th1 cytokines. This change could be accomplished by either a quantitative reduction in allergen-specific Th2 responses or the selective induction of Th1 cytokines by allergen-reactive Th0 cells, both present in the T cell repertoire of atopic individuals (25). This prompted us to analyze peptide analogues of *Der p* II, residues 28 to 40, for their antigenicity and ability to stimulate cytokine production by DRB1*1101-restricted T cell clones representative of the Th0 and Th2 functional phenotypes (20).

The substitution of single amino acids with alanine at each position in p28–40 had similar effects on the pattern of proliferation of both Th0- and Th2-specific cells and markedly reduced the response only when introduced at each of three positions (31, 32, and 33). In contrast, analogues containing positively and negatively charged amino acid substitutions had consistently negative effects on Ag recognition, although this was less pronounced when the substituted natural amino acid was of a similar charge. These results taken together with the MHC-peptide binding studies suggest that the residues at positions 31 and 32 are critical for TCR recognition. The capacity of analogues with different amino acid substitutions at position 33 to bind with varying affinities to class II MHC implies that this position makes contact with DRB1*1101. However, the residue at this position may also contact TCR, since the analogue A^{33} appears to bind to DRB1*1101 with an affinity similar to that of the native peptide, but is less antigenic. These conclusions are consistent with earlier findings suggesting that for both AC1 and AC2 T cells, the peptide core for TCR recognition may be located between residues 28 and 35, whereas sequences 22 to 28 or 35 to 40 may be implicated mainly in binding to HLA-D region molecules (20). Results from the functional competition assays imply that, in addition to residue 33, those amino acids at positions 29, 30, 35, and 38 in p28–40 contribute to peptide binding to DRB1*1101. These residues either directly contact MHC or influence the binding capacity of the peptide indirectly by altering the conformation of the side chains of adjacent amino acids. For peptides bound to HLA-DRB1*1101, a potential motif has been suggested, consisting of three main anchoring positions, an aromatic residue at position 1 followed by a hydrophobic and a positively charged amino acid at positions 4 and 6, respectively (26, 27). Assuming that the aromatic residue H^{30} in p28–40 corresponds to the relative position 1, the residues implicated in MHC binding found at positions 4 and 6 would be K^{33} and F^{35}. However, although the residues at these positions appear to contact MHC, their natures would not comply with the proposed binding motif for DRB1*1101, and this may account for the ability of *Der p* II 28–40 to bind to other class II molecules, including HLA-DQ (20). MHC class II-restricted T cell recognition of peptides not exhibiting the binding requirements for that particular MHC class II specificity have been described (28).

Rules analogous to those governing MHC class II-peptide binding also appear to influence the outcome of TCR interactions with altered peptide ligands. It has been demonstrated that analogues of an antigenic peptide with the primary TCR contact unaltered but containing selected substitutions at secondary TCR contact residues could act as agonists or partial agonists for specific Th cells (13, 14). Furthermore, unrelated peptides with sequence similarities at these positions were found to be capable of eliciting similar functional activity. Nevertheless, TCR recognition remains a uniquely specific phenomenon, since Th cells with different fine specificities presented a variable pattern of responsiveness to the same ligands (29).

In addition to the effects of alterations in the structure of the p28–40 peptide on Ag recognition, the effects on cytokine production by both Th0 cells and Th2 cells were analyzed. Despite the similarity in the proliferative responses of both subsets of specific Th cells to the p28–40 analogues, the pattern of IL-4 secretion varied markedly. In response to the p28–40 analogues, Th0 cells induced IL-4 production, which generally reflected the pattern of clonal expansion, although minor quantitative differences were noted. In contrast, stimulation of Th2 cells with selected p28–40 analogues induced IL-4 secretion in the absence of proliferation. Although differences in the fine specificity of the two T cell clones may account for this disparity, it cannot be excluded that stimulation with the altered T cell ligands may have different effects on the signaling pathways that induce IL-4 production in both Th0 and Th2 cells. Stimulation of Th0 cells with p28–40 analogues containing alanine at position 34 or 36 resulted in a selective enhancement of IFN-γ secretion that was independent of IL-4 production and the degree of proliferation. Previous reports have demonstrated that proliferation and cytokine production in murine Th2 cells have distinct thresholds of induction and can be selectively triggered (16). In particular, IL-4 synthesis appears to be favored by signals provided by less potent TCR-ligand interactions, as in the case of reduced ligand density (30) or by stimulation with peptides of low affinity for TCR or MHC class II (13, 17, 18). Furthermore, IL-4 secretion by Th2 cells seems to have less stringent requirements for coreceptor or accessory molecule-mediated costimulation. It has been observed that murine Th2 cells retained their capacity to secrete IL-4 when stimulated by specific peptide on chemically fixed APCs (16). It appears also that certain anti-CD4 Abs that inhibited Ag-dependent proliferation of p28–40-specific Th2 cells failed to down-regulate IL-4 secretion (23). Moreover, when activated in the absence of costimulatory signals from APCs, Th0 cells adopted the cytokine profile of Th2 cells, implying that the Th2 phenotype correlates with the induction of anergy in Th0 cells (31).

The potential use of altered T cell ligands to modulate the effector function of Th2 cells may be of limited value, since selective inhibition of IL-4 production has not been achieved. However, when *Der p* II-specific Th0 cells were stimulated with p28–40 analogues (A^{34} and A^{36}), IFN-γ production was enhanced fivefold compared with that of the native ligand, whereas IL-4 production remained unchanged. The importance of regulating the balance between Th1- and Th2-derived cytokines was demonstrated in a recent clinical study, in which it was reported that successful desensitization with bee venom was accompanied by alterations in the IFN-γ:IL-4 ratios (32). Increased expression of mRNA for Th1-type cytokines at the site of late phase cutaneous reactions was described in successfully treated individuals (4). Therefore, an alternative strategy to beneficially intervene in atopic disorders

may be by targeting Th0 cells in an attempt to alter the IL-4:IFN-γ ratio in favor of IFN-γ.

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Regulation of cytokine production by human Th0 cells following stimulation with peptide analogues: differential expression of TGF-β in activation and anergy

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SUMMARY

The different biological activities of T-cell-derived cytokines and their level of production influences the qualitative nature of immune responses and, in certain forms of T-cell tolerance, the lack of antigen responsiveness is associated with the production of transforming growth factor-β (TGF-β) and interleukin-4 (IL-4). In this study we have investigated the effects of T-cell receptor (TCR) ligation with peptide analogues and the native peptide, in the presence and absence of costimulation, on cytokine production by human T-helper type 0 (Th0) cells reactive with influenza virus haemagglutinin (HA) peptide (HA306–318) and restricted by HLA-DRB1*0101. We observed that resting Th0 cells constitutively produced TGF-β, but when stimulated with peptide and antigen-presenting cells (APC) under conditions that induce clonal expansion, TGF-β secretion was abrogated. Furthermore, exposure of the T cells to the wild-type HA peptide under conditions that induce T-cell anergy resulted in the secretion of TGF-β, and subsequent antigenic rechallenge was unable to override this signal and down-regulate TGF-β production. Stimulation with altered TCR ligands that failed to induce proliferation also resulted in marked production of TGF-β, although in many instances the levels were less than those observed in the total absence of antigen, suggesting that partial signalling has occurred. Although in general, there was a direct positive correlation between proliferation and the production of IL-2, IL-4 and interferon-γ (IFN-γ) following stimulation with certain analogues, the production of selected cytokines was dissociated.

INTRODUCTION

$CD4^+$ T cells may be divided into functional subsets with distinct cytokine secretion profiles and, therefore, exhibit different biological properties, which contribute to the qualitative nature of immune responses (reviewed in ref. 1). In a variety of experimental models it has been demonstrated that the activation of T-helper (Th) cells representative of a particular functional phenotype may be necessary for generating effective immunity.[2–4] In contrast, either failure to secrete cytokines with the required biological functions or expansion of Th cell subsets eliciting inappropriate activities may result in immunopathological reactions, such as those observed in autoimmunity, allergic disorders and chronic infections (reviewed in ref. 5). Consequently, the potential to induce or inhibit the production of cytokines derived from specific Th cells in order to promote protective immunity in vaccination or to down-regulate pathogenic responses in immunotherapy has attracted considerable interest.[6,7]

There is now evidence from murine systems suggesting that modulation of T-cell effector functions and selective cytokine production can be achieved by changing the affinity of the interaction between T-cell receptor (TCR) and its cognate ligand[8–11] or, alternatively, through the induction of regulatory T cells.[12,13] The failure of T cells to proliferate on antigenic restimulation following ligation of the TCR with native peptide[14,15] or altered T-cell ligands[11,16] in either the presence or absence of costimulation is, in many instances, associated with dysregulation of cytokine production.[15,17–19] In general, the secretion of Th1-type cytokines appears to be readily inhibited in anergy, whereas Th2 and Th0 cells retain the ability to secrete selected cytokines, such as interleukin-4 (IL-4), even though their capacity to proliferate on antigen restimulation is abrogated.[18,19] Furthermore, the analysis of the cellular basis of oral tolerance has revealed that both $CD8^+$ and $CD4^+$ T cells capable of synthesizing transforming growth factor-β (TGF-β) as well as IL-4, may be induced.[12,13] In this model it is proposed that the negative effects of IL-4 are elicited through the cross-inhibition of Th1 cells, whereas TGF-β has more generalized immunosuppressive activities.

Collectively these observations prompted us to investigate the effect on cytokine production by human Th0 cells on stimulation, in the presence of antigen-presenting cells (APC), with wild-type peptide [influenza haemagglutinin (HA) residues 306–318] and HA analogues in which the TCR contact

Received 25 March 1997; revised 14 May 1997; accepted 14 May 1997.

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residues had been substituted. The production of IL-2, IL-4, interferon-γ (IFN-γ) and TGF-β was studied in relation to clonal expansion. In addition, we examined the ability of Th cells to secrete TGF-β in absence of costimulatory signals during the induction phase of anergy and on restimulation of the anergic T cells with native peptide under immunogenic conditions.

MATERIALS AND METHODS

Antigens

The influenza virus haemagglutinin peptide residues 306–318 (PKYVKQNTLKLAT) and analogues with substitutions at the TCR contact residues were synthesized on a Multipeptide Synthesis block BT7400 (Cambridge Research Biochemicals, Northwich, UK) using 4-(2′, 4′ dimethoxyphenyl FMOC aminomethyl) phenoxy resins (Novasyn Chemicals Ltd, Nottingham, UK). Couplings were performed using FMOC side chain-protected pentaflurophenyl or oxo-benzoteiazine amino acid esters. Full-length peptides were cleaved from the resin and deblocked by treatment with trifluroacetic acid/thioanisole/ethanedithiol (94/5/2·5 v/v) for 1 hr, precipitated and then washed twice with ether. The precipitates were dissolved in 10% formic acid (v/v) and freeze dried for 48 hr. Each peptide produced a single peak by reverse-phase high-performance liquid chromatography (HPLC). The peptide analogues used in this study are listed in Table 1.

Table 1. Amino acid sequences of peptide analogues of HA 306–318

	307		309	310		312			315			
P	K	Y	V	K	Q	N	T	L	K	L	A	T
	E											
	F											
	H											
	Q											
	R											
			A									
			D									
			F									
			G									
			H									
			K									
			P									
				A								
				D								
				Q								
				R								
				S								
						A						
						F						
						G						
						H						
						P						
						Q						
									A			
									F			
									G			
									H			
									S			
									V			

*Isolation of HA 306–318-reactive DRB1*0101-restricted cloned human Th0 cells*

T cells of HA1.7 were isolated by limiting dilution cloning as described previously.[14] The T-cell clone was maintained in RPMI-1640 medium supplemented with 2 mM L-glutamine, 100 IU/ml penicillin/streptomycin (Gibco, Life Technologies, Paisley, UK) and 5% inactivated human A^+ serum (National Blood Transfusion Service, Edgware, UK) by stimulation with the HA 306–318 peptide and irradiated histocompatible peripheral blood mononuclear cells (PBMC) (5×10^5/ml; 3000 rads) as a source of APC, together with IL-2 (10% v/v; Lymphocult T, Biotest Folex, Frankfurt, Germany). In all experiments, clones were tested 7–8 days after the last addition of antigen and APC.

Proliferation assays

Cloned T cells (HA1.7; $2{\cdot}0 \times 10^4$/well) were cultured in flat-bottomed 96-well microtitre trays (Nunc, Gibco Life Technologies) with analogues of HA 306–318 (3 or 10 μg/ml) in the presence of APC. Mitomycin C-treated murine fibroblasts expressing HLA-DRB1*0101 (2×10^4 cells/well) were used for antigen presentation. In some experiments irradiated histocompatible PMBC ($2{\cdot}5 \times 10^4$ cells/well) or Epstein–Barr virus (EBV)-transformed B cells (2×10^4 cells/well) were used as APC. Cultures were pulsed with tritiated thymidine (1 μCi/well; [^{3}H]TdR; Amersham International Plc, Amersham, UK) after 48 hr and harvested 8–16 hr later. Proliferation was determined by [^{3}H]TdR incorporation by liquid scintillation spectroscopy. The results are expressed as mean c.p.m. for triplicate cultures with SEM < 20%.

Induction of T-cell anergy

T cells (10^6/ml) were cultured in the presence of peptide (100 μg/ml) or in medium alone for 18 hr and supernatants collected for the measurement of TGF-β. The T cells were then washed extensively before being rechallenged with either an optimal concentration of native peptide in the presence of APC (DR1-expressing murine fibroblasts) or IL-2 alone, as described previously.[14] Supernatants were also collected from these cultures for the measurement of TGF-β. Proliferation was measured by the incorporation of ^{3}[H]TdR.

Measurement of IL-2, IL-4, IFN-γ and TGF-β

Supernatants were collected at 48 hr from cultures of HA1.7 cells stimulated with peptides in the presence of APC. Triplicate wells were pooled and IL-2 was measured using the CTLL-2 line, whereas IFN-γ and IL-4 were measured by enzyme-linked immunosorbent assay (ELISA) kits.[11] TGF-β was assayed using the cell line Mv 1 Lu (NBL-7), as described previously.[20] TGF-β was measured in supernatants collected during the induction phase of anergy and following antigenic restimulation of the anergic T cells as described above.

RESULTS

The effect of amino acid substitutions at the TCR contact residues in the HA peptide 306–318 on cytokine (IL-2, IL-4, IFN-γ and TGF-β) production by human Th0 cells

Resolution of the crystal structure of DRB1*0101/HA 306–318 complexes suggests that residues at positions 307, 309, 310, 312, 315 and 318 may interact with the TCR.[21] Therefore,

peptide analogues with substitutions at these positions were synthesized (Table 1) and analysed for their ability to induce selected T-cell effector functions and/or anergy, with the exception of 318, since we have reported in a previous study that threonine (318) is not required for recognition of the HA peptide by HA 1.7 cells.[22] Initially the activity of the HA analogues was tested in T-cell proliferation assays and, subsequently, analogues with different potencies were analysed for their ability to induce IL-2, IL-4, IFN-γ and TGF-β production or anergy in the absence of costimulation.

Substitutions at position 307. The analogue R^{307} stimulated proliferation similar in magnitude to the wild-type HA peptide (K^{307}), whereas for F^{307}, H^{307} and Q^{307} proliferation was reduced to the order of 50% (Fig. 1a). In general, the production of TGF-β by HA1.7 cells was inversely correlated with proliferation, such that native peptide and R^{307}, which stimulated clonal expansion, failed to induce TGF-β production (Fig. 1b). In cultures of the T cells with APC and E^{307} or APC alone, the highest levels of TGF-β were detected. In order to exclude the possibility that incomplete costimulation was being provided by the HLA-DR1-transfected murine fibroblasts and that human accessory molecules were required for TGF-β production by the HA 1.7 cells, histocompatible PBMC or EBV-transformed B cells were used as a source of APC. In the presence of additional costimulation similar results were observed and proliferating T cells failed to secrete TGF-β (data not shown). In contrast to TGF-β, IL-4 (Fig. 1c) and IFN-γ (Fig. 1d) production paralleled the proliferative response, but IL-2 was only detected in the supernatant of cultures stimulated with wild-type peptide and R^{307} (Fig. 1e). TGF-β production was measured at 48 hr; however, in kinetic studies sampling supernatants at 24, 48 and 72 hr an inverse relationship between proliferation and TFG-β was still observed. We have observed that other Th0 cells, in addition to HA1.7 cells, constitutively produce TGF-β, which is abrogated by antigenic stimulation (results not shown).

Substitutions at position 309. The analogues with Ala, Gly, His or Pro at position 309 induced proliferation but at a lower level than the native peptide, V^{309} (Fig. 2a). Minimal proliferation was observed by T cells stimulated with F^{309} or K^{309}. Of the analogues tested, A^{309}, G^{309}, H^{309}, P^{309} and V^{309} all failed to induce the synthesis of TGF-β (Fig. 2b). In contrast, neither proliferation nor TGF-β production was induced by F^{309}. IL-4 levels were comparable following stimulation with G^{309}, H^{309} and V^{309}, whereas for P^{309}, A^{309} and D^{309} levels were reduced by 22%, 75% and 87%, respectively, and no IL-4 was induced by F^{309} or K^{309}. Minimal levels or no detectable IFN-γ was found in cultures stimulated with the analogues P^{309}, D^{309}, F^{309} or K^{309}. However, for A^{309} and G^{309} the levels were equal to or greater than V^{309} (Fig. 2d). IL-2 production had a similar response profile to IFN-γ, except that D^{309}, F^{309} and K^{309} induced very low but detectable levels (Fig. 2e).

Substitutions at position 310. The amino acid at position 310 appears to be a primary TCR contact residue in that all the analogues tested, with the exception of the conservative substitution of Arg for Lys, ablated the effector functions of HA1.7 cells (Fig. 3). However, despite their inability to induce proliferation (Fig. 3a) or the production of IL-2 (Fig. 3e), IL-4 (Fig. 3c) and IFN-γ (Fig. 3d) in the presence of the analogues A^{310}, Q^{310} and S^{310}, the T cells secreted TGF-β, although at reduced levels compared to D^{310} and the medium control (Fig. 3b).

Substitutions at position 312. Analogues with the naturally occurring residue Asn at position 312 substituted with His, Ala, Gly, or Phe stimulated T-cell proliferation, although reduced in magnitude (Fig. 4a) compared to the native peptide, while in response to H^{312} the proliferation was marginally increased (14%). No proliferation of the HA1.7 cells was induced by Q^{312} or P^{312}, but both of these analogues stimulated the production of TGF-β (Fig. 4b), although the amount secreted was less than the constitutive production. Low levels of TGF-β were detected in cultures of the T cells with A^{312} and H^{312}. For IL-4 (Fig. 4c), IFN-γ (Fig. 4d) and IL-2 (Fig. 4e), in general cytokine production reflected clonal expansion. Nevertheless, stimulation with A^{312} induced enhanced IFN-γ (Fig. 4d) and reduced IL-2 (Fig. 4e) secretion compared to the natural ligand.

Substitutions at position 315. All the analogues tested with substitutions at positions 315 stimulated less proliferation (Fig. 5a) than the native peptide (K^{315}). TGF-β production was inversely correlated with the proliferative response (Fig. 5b). IL-4 (Fig. 5c), IFN-γ (Fig. 5d) and IL-2 (Fig. 5e) secretion mirrored proliferation, except for S^{315} which failed to stimulate levels of IL-4 or IFN-γ that were detectable in the culture supernatants.

TGF-β production by anergic Th0 cells

We have previously reported that HA1.7 cells exposed to supraimmunogenic concentrations of native peptide or analogues in the absence of costimulation become anergic to restimulation with antigen.[11,14] These studies have been extended to investigate the effects of high doses of wild-type peptide and selected analogues on TGF-β secretion during the induction phase of anergy and when anergic T cells are restimulated (Fig. 6). Exposure of HA1.7 cells to native peptide (100 μg/ml) induced TGF-β release, and on rechallenge of the anergic cells with an antigenic concentration of the wild-type peptide in the presence of APC the cytokine was still produced. Pretreatment of HA1.7 cells with F^{307}, H^{307}, H^{309}, G^{312} and H^{315} had similar effects to the native peptide. K^{312} also induced a similar pattern of TGF-β production but reduced in magnitude. Exposure to the analogues Q^{312} and P^{312} in the absence of APC induced some TGF-β release, similar to the medium control, but on restimulation no further production of TGF-β was observed.

DISCUSSION

The results of this study provide further evidence that selective cytokine production by human Th0 cells can be achieved by modulating the conditions of T-cell activation, namely by using altered peptide ligands or inducing T-cell anergy. This analysis is an extension of our previous work in which the effects of altered T-cell ligands on proliferation and cytokine production, namely IL-2, IL-4 and IFN-γ, during the induction of T-cell anergy was investigated.[11] Here we report that certain human Th0 cells constitutively produce TGF-β and that this secretion is inhibited when the T cells are stimulated with antigenic concentrations of peptide in the presence of APC. The use of peptide analogues with altered affinities for the TCR also revealed an inverse correlation between the ability to induce clonal expansion and TGF-β production. In contrast,

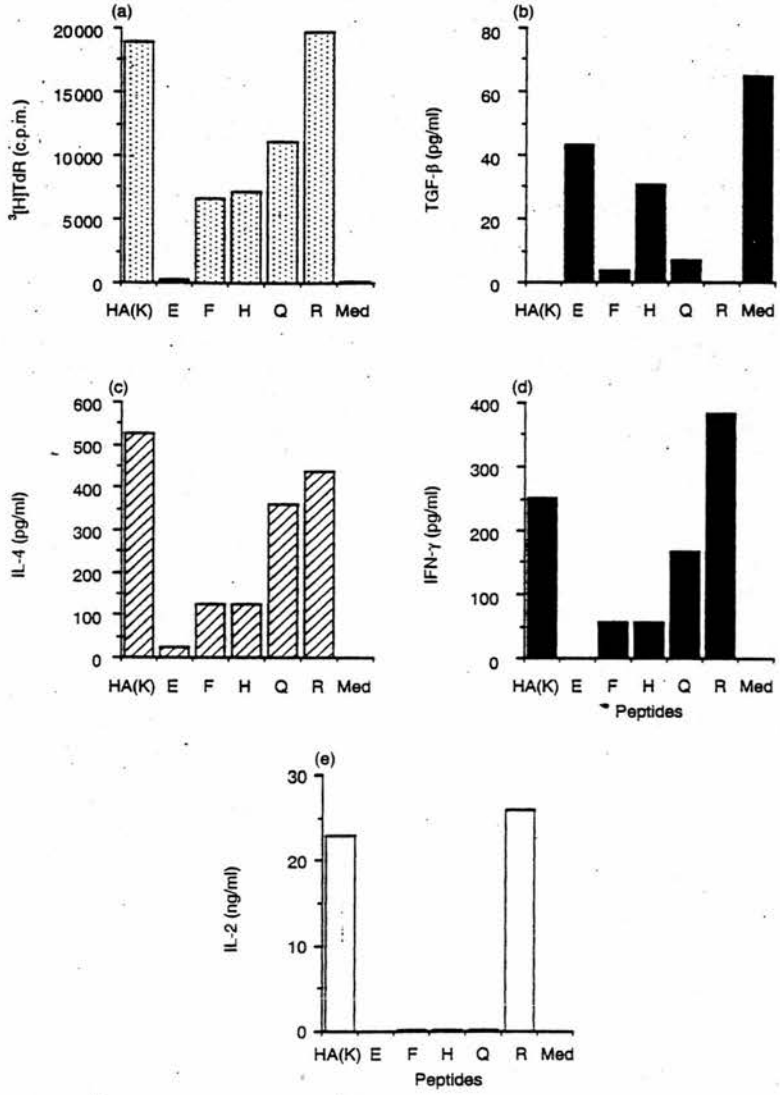

Figure 1. Effects of amino acid substitutions at position 307 in HA 306–318 on T-cell proliferation and the induction of cytokines. (a) cloned T cells were stimulated with the HA^{307} analogues indicated at 3 and 10 μg/ml in the presence of APC (mitomycin C-treated HLA-DRB1*0101 expressing murine fibroblasts). 3[H]TdR incorporation was determined at 72 hr. The response of cultures containing 3 μg/ml of peptide are presented. The background proliferation of T cells cultured with APC in the absence of peptide was measured (Med). HA1.7 cells were cultured with peptide analogues in the presence of APC and culture supernatants were harvested at 48 hr for the measurement of (b) TGF-β, (c) IL-4, (d) IFN-γ and (e) IL-2.

IL-2, IL-4 and IFN-γ synthesis occurred only after TCR ligation and, for the majority of the HA analogues, the amount of cytokine produced paralleled the magnitude of proliferation. However, for selective analogues, such as A^{309} and A^{312}, differences in the ratio of IL-4 to IFN-γ favouring the Th1 pathway were observed, while in response to P^{309} preferential secretion of IL-4 without significant amounts of IFN-γ was noticed. Anergy induced by exposure of the T cells to supra-immunogenic concentrations of wild-type peptide and selected analogues in the absence of costimulation resulted in TGF-β production that was not overridden by antigenic restimulation.

The production of TGF-β by resting and anergic HA1.7

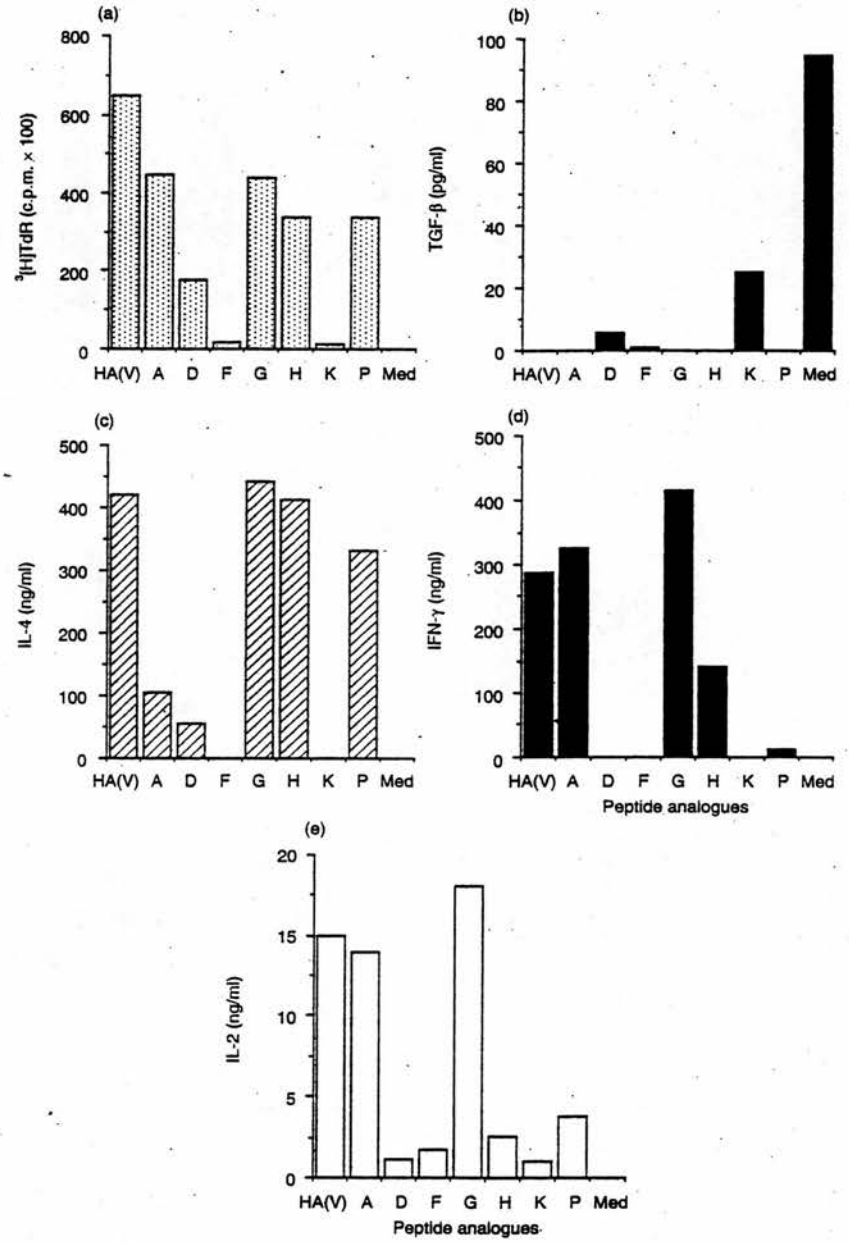

Figure 2. Effects of amino acid substitutions at position 309 in HA 306–318 on T-cell proliferation and the induction of cytokines. The HA^{309} analogues examined are indicated on the figure. The results presented are as described in the legend to Fig. 1.

cells may be associated with down-regulation of the IL-2 signalling pathway that characterizes both states.[15] Antagonism between the actions of the two cytokines has been documented.[23] Furthermore, it has been shown that the mRNA for TGF-β and IL-2 is differentially regulated in human T cells, with the immunosuppressant cyclosporine A selectively inducing mRNA specific for TGF-β and down-regulating that for IL-2.[24] Secretion of TGF-β from resting

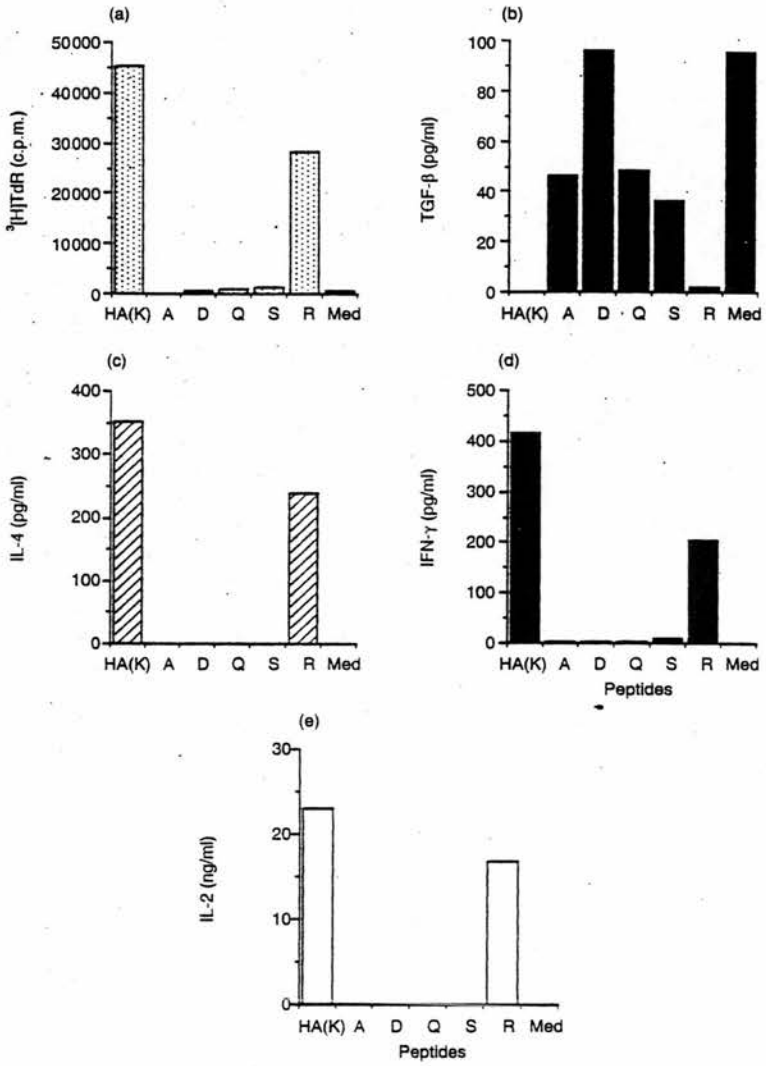

Figure 3. Effects of amino acid substitutions at position 310 in HA 306–318 on T-cell proliferation and the induction of cytokines. The HA^{310} analogues examined are indicated on the figure. The results presented are as described in the legend to Fig. 1.

T cells in the absence of TCR-mediated stimulation may also be triggered by IL-2 through translational and/or post translational mechanisms.[25] TGF-β reported to be a potent inhibitor of cell grwoth by blocking cell cycle transition from G1 to S phase (reviewed in ref. 26). As regards CD4$^+$ T cells, TGF-β can suppress proliferation as well as effector functions, including Th1- and Th2-type cytokine synthesis,[27,28] although under certain conditions stimulatory effects have been observed.[29] In an attempt to explain these contradictory findings it has been suggested that the effects of TGF-β may depend on the maturation stage of the T cells and their functional characteristics and activation requirements, such as the nature of costimulatory signals required for clonal expansion.[30]

In general, in response to altered T-cell ligands the HA1·7 cells produced TGF-β only when significant proliferation was not included. However, quantitative differences in the levels of TGF-β between unstimulated T-cell cultures and those containing the non-stimulatory HA analogues were observed, e.g. K^{309} and H^{315}. Furthermore, stimulation with H^{307} and H^{312} induced both TGF-β release and proliferation, whereas the analogues F^{309} and A^{315}, which are non-antigenic, failed to trigger TGF-β synthesis. The disparities may be due to

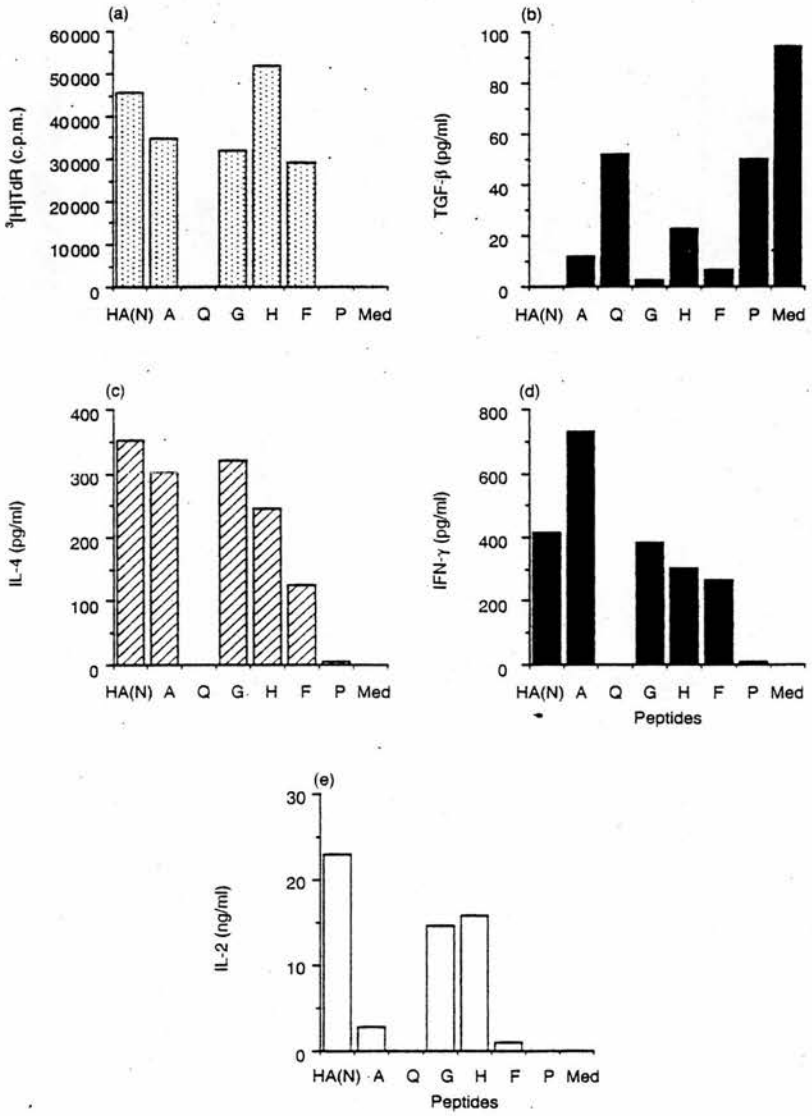

Figure 4. Effects of amino acid substitutions at position 312 in HA 306–318 on T-cell proliferation and the induction of cytokines. The HA^{312} analogues examined are indicated on the figure. The results presented are as described in the legend to Fig. 1.

partial agonist effects of the analogues resulting in the activation of some but not all of the effector functions of the T cells. These findings suggest that pathways regulating TGF-β production may also be affected by alterations in the intracellular signalling generated by lowering the affinity of TCR/ligand interactions. It has recently been reported that stimulation of cloned myelin basic protein (residues 85–99)-reactive Th0 cells with a partial agonist peptide resulted in selective TGF-β production.[31] However, the authors considered TGF-β production to be a consequence of the action of the peptide analogue and not due to the intrinsic ability of the specific Th0 cells to generate TGF-β in the absence of full activation signalling, although low levels of secretion of the cytokine by resting cells was observed.

In contrast to TGF-β, the amounts of IL-2, IL-4 and IFN-γ present in supernatants collected from cultures of the Th cells stimulated with the HA analogues reflected, in general, the degree of clonal expansion. None the less, in response to

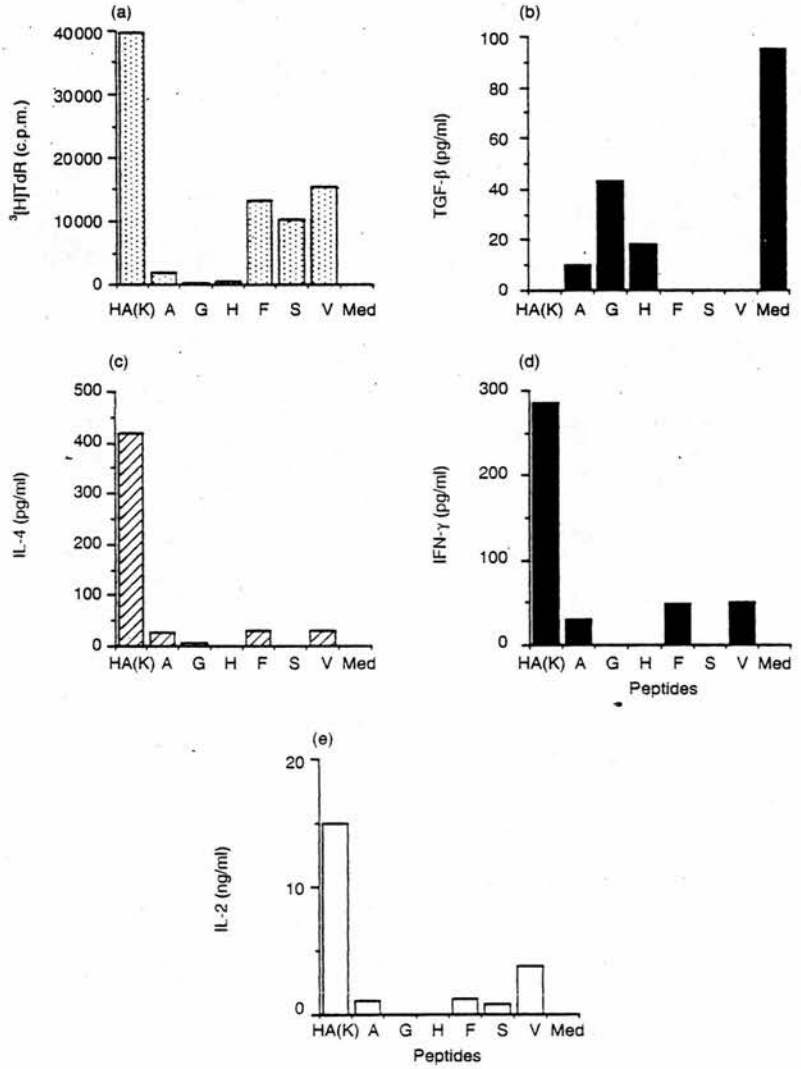

Figure 5. Effects of amino acid substitutions at position 315 in HA 306–318 on T-cell proliferation and the induction of cytokines. The HA^{315} analogues examined are indicated on the figure. The results presented are as described in the legend to Fig. 1.

certain analogues the cytokine production was dissociated from proliferation. IL-2 was not detected in some T-cell cultures (e.g. F^{312}), although proliferation and secretion of both IFN-γ and IL-4 occurred. Levels of IL-2 disproportionate to the magnitude of the proliferative response may result from a failure of the HA analogue to induce IL-2 production or enhanced up-regulation of IL-2 receptors leading to increased IL-2 consumption. Studies in which T cells were stimulated with anti-TCR antibody[32] or a mutated MHC–peptide ligand[10] have revealed that the pathways for activation of IL-2 secretion and CD25 expression can be dissociated.

Quantitative differences were noticed in the levels of IFN-γ and IL-4 secreted by the T cells in response to selected analogues, e.g. A^{309} induced low levels of IL-4 with levels of IFN-γ similar to the wild-type peptide, A^{312} selectively triggered enhanced IFN-γ production, while P^{309} failed to stimulate secretion of IFN-γ although the synthesis of IL-4 remained intact. These results suggest that the effector activities of Th0 cells have different thresholds and can be triggered either selectively or quantitatively. The signalling pathways that regulate Th1-type cytokine production overall appear to be more sensitive to the effects of altered peptide ligands than

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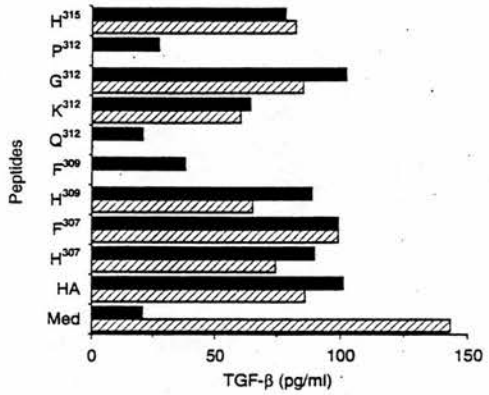

Figure 6. TGF-β production by Th0 cells during the induction phase of anergy and when anergic T cells are subjected to immunogenic restimulation. HA1.7 cells were stimulated with native peptide and selected analogues in the absence of APC and the supernatants collected at 18 hr for the measurement of TGF-β (stippled bars). The T cells were washed and then restimulated with APC and native peptide and TGF-β was measured in supernatants collected at 48 hr (cross-hatched bars).

those of IL-4 and this difference may result from IL-2, IL-4 and IFN-γ gene transcription being under the control of separate regulatory elements. It has recently been demonstrated that activation of both NF-ATp and AP-1 is required for Th1 cytokines gene induction, whereas the IL-4 gene involves only NF-ATp.[33] Moreover, the differential effect of activation of the protein kinase A pathway on the expression of IL-2 and IL-4 gene was shown to be mediated by a site in the IL-2 promoter, which is absent from the IL-4 promoter.[34] The intracellular signalling pathways leading to secretion of Th1 and Th2 cytokines also have different sensitivities to inhibitory stimuli, such as immunosuppressive agents[35] or anergy induction.[18,19]

Ligation of TCR with peptide ligands in the absence of costimulation results in the induction of T-cell anergy.[11,14,15] Human cloned Th0 cells exposed to high concentrations of the native peptide and selected analogues, in the absence of APC, exhibit dysregulated cytokine production and fail to proliferate on antigenic restimulation.[17] In this study we report that during the induction phase of anergy TGF-β was released by T cells incubated with the wild-type peptide or analogues capable of inducing anergy (e.g. H^{307} and H^{309}). When the anergized T cells were rechallenged with the native peptide in the presence of APC they retained their ability to secrete TGF-β. The delivery of an antigenic signal to the anergic T cells was unable to override TGF-β production initiated during the induction phase of anergy. Reduced levels of TGF-β, similar to those generated in the control cultures, were also noticed for T cells exposed to analogues that were unable to induce anergy (e.g. Q^{312}). However, restimulation of these T cells under immunogenic conditions resulted in inhibition of the cytokine production. This observation implies that although the HA1.7 cells can produce TGF-β in the absence of a signal inducing proliferation, this production can be further enhanced by an anergic stimuli. In models of oral tolerance it has been demonstrated that T cells secreting TGF-β alone or together with IL-4 and IL-10 are induced and that these cells are capable of actively suppressing specific responses.[12,13,36] Furthermore, it has been concluded that these T-cell populations constitute an independent Th cell subset, termed Th3. However, certain anergic Th0 cells could also have the same functional phenotype.

Clearly the ability to alter the affinity of TCR–ligand interactions and promote the production of growth inhibitory and anti-inflammatory cytokines may be relevant to the development of specific immunotherapy for allergic and autoimmune diseases.

ACKNOWLEDGMENTS

This work was supported by grants from the Wellcome Trust, UK, and the Medical Research Council, UK. The authors thank Ms Hailing Xu for secretarial assistance.

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2.3 ANTIGEN MEDIATED MODULATION OF T CELL FUNCTION

2.3.3 ANALYSIS OF *IN VITRO* ANTIGEN-INDUCED SPECIFIC ANERGY

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INDUCTION OF TOLERANCE IN INFLUENZA VIRUS-IMMUNE T LYMPHOCYTE CLONES WITH SYNTHETIC PEPTIDES OF INFLUENZA HEMAGGLUTININ*

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T cell clones have been highly useful for investigating various aspects of T cell-mediated immunity and immunoregulation. For example, they have been useful for analyzing the genetic restriction of lymphoid cell interactions, soluble mediators of T cell function, and the T cell repertoire (reviewed in references 1 and 2). Furthermore, T cell clones have also been useful as homogeneous populations to examine mechanisms of T cell activation (1–4), and also offer the opportunity to examine regulation of the immune system in more detail. Thus we have recently developed a suppressor cell clone that specifically recognizes an antigen-specific helper cell and inhibits its helper function (5). This will permit a detailed analysis of regulation by suppressor effector cells.

Regulation of the immune response by antigen is a well documented phenomenon. Mitchison (6) reported that antigen administered in either supraoptimal concentrations or repeated suboptimal doses, may induce the state of antigen-specific, antigen-induced unresponsiveness commonly termed immunological tolerance. Further analysis by Chiller and Weigle (8), Mitchison (7), Rajewsky (9), and others located the cellular sites of unresponsiveness and revealed that although B cell tolerance occurred transiently, tolerance of T cells was more persistent and was the major lesion in high zone tolerance and the only one in low zone tolerance (7–9).

Further research into the cellular mechanisms of tolerance has involved the analysis of tolerance induced in vitro. Thus, Diener and Feldmann (10, 11) found that B cell tolerance was induced in vitro by incubation with a high dose of antigen for 3–6 h at 37°C, a process that depended critically on the nature of the antigen used and its polymeric interaction with B cell surface receptors. This process was reversible with enzyme treatment for the first few days (12). In contrast, the induction of T cell tolerance in vitro has not been reproducible, so its molecular basis has been difficult to study. However, the cellular mechanism of tolerance has been investigated in vivo, and it has been suggested that suppressor T cells play a critical role, especially in T

* Supported by contract N000-14-77C-0748 from the U. S. Office of Naval Research.

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J. Exp. Med. © The Rockefeller University Press · 0022-1007/83/05/1434/14 $1.00
Volume 157 May 1983 1434–1447

cell tolerance (13–16), although suppressor cells were not always detected, and when found, did not correlate exactly with the tolerant state.

These results however, did not exclude the possibility that T cell tolerance could occur in the absence of suppressor cells (e.g., 17, 18). The existence of cloned populations of helper T cells offers the opportunity of determining whether antigen, by itself, can tolerize helper T cells in the absence of suppressor T cells or their precursors. The present report indicates that brief incubation with synthetic peptides of the hemagglutinin (HA)[1] molecule may specifically inhibit the proliferation of cloned human helper T cells, suggesting that tolerance can be induced by high concentrations of antigen without the mediation of suppressor T cells.

Materials and Methods

Antigens. Formalin-inactivated influenza viruses of the strains A/Texas (A/Texas/1/77X49(H_3N_2); lot 53142) and B/Singapore (B/Sing) (B/Singapore/222/79; lot 71719) were obtained from Merck Sharp & Dohme, Rahway, NJ. Immunochemically purified influenza A virus HA (A/Bangkok/1/79; H_3N_2) was generously provided by Dr R. G. Webster, St. Jude Children's Research Hospital, Memphis, TN. The peptides of the HA1 molecule of influenza hemagglutinin were synthesized according to the amino acid sequence of A/Hong/Kong/X47 (H_3N_2) (19) as predicted from the nucleotide sequence (20). In this study peptides 4 (amino acid sequence 39–65), 11 (105–140), and 20 (306–329) were used.

Lymphocyte Preparation and Fractionation. The preparation and fractionation of peripheral blood mononuclear leukoytes (PBL) have been described in detail elsewhere (21, 22). Briefly, cryopreserved PBL from a healthy adult volunteer isolated on a Ficoll-Hypaque density gradient were used throughout these experiments. T cell-enriched populations (E^+) were isolated from PBL by rosetting with S-2-aminoethylisothiouronium bromide hydrobromide (AET) (Calbiochem-Behring Corp., San Diego, CA)-treated sheep erythrocytes. After centrifugation over Percoll (d = 1.080 g/ml; Pharmacia Fine Chemicals, Uppsala, Sweden), the non-rosette-forming cell (E^-) fraction, which contained <1% E^+ cells, was recovered from the interface and the E^+ fraction was recovered from the pellet by lysis of the erythrocytes with Gey's hemolytic solution.

Production of T Cell Growth Factor (TCGF). TCGF was prepared by culturing normal human PBL (1×10^6/ml) with 0.1% purified phytohemagglutinin (PHA) (Difco Laboratories, Detroit, MI) in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 2 mM L-glutamine, 1 mM Na pyruvate, 10 IU/ml Na heparin, 25 mM Hepes, and 50 μg/ml gentamicin, and containing 1% autologous plasma (23). After 48 h, supernatants were harvested and assayed for their ability to support the growth of a TCGF-dependent cell line (22).

Isolation of Antigen-specific T Lymphocyte Clones (TLC). Antigen-specific TLC were isolated as described previously (22). Briefly, normal PBL (2.5×10^5/ml) were cultured with purified HA (0.1 μg/ml) in supplemented RPMI 1640 containing 10% screened pooled A^+ serum. At 6 d the lymphoblasts were enriched on a 35–40% discontinuous Percoll gradient, resuspended at 33⅓ cells/ml and plated at one cell every third well in Microtest II trays (Falcon Division, Becton, Dickinson & Co., Cockeysville, MD) with 10^4 irradiated (2,500 rad) autologous PBL and HA (0.1 μg/ml) in the presence of 20% TCGF. After 7 d, growing clones were transferred to flat-bottomed 96-well microtiter trays (Costar, Data Packaging, Cambridge, MA) and subsequently to 24-well trays (Costar). At each transfer the TLC received fresh TCGF (20%) and irradiated autologous PBL (5×10^5/ml) together with specific antigen (HA; 0.1 μg/ml). TLC were maintained with fresh TCGF every 3–4 d and irradiated autologous PBL and HA were added every 7 d. Before use in proliferation or helper assays the TLC were rested 7 d after the addition

[1] *Abbreviations used in this paper:* AET, S-2-aminoethylisothiouronium bromide hydrobromide; BSA, bovine serum albumin; B/Sing, B/Singapore; DNP, dinitrophenyl; E^+, sheep erythrocyte rosette-forming lymphocytes; E^-, sheep erythrocyte non-rosette-forming lymphocytes; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; HA, hemagglutinin; HAU, hemagglutinating units; [^{3}H]TdR, tritiated methyl thymidine; PBL, peripheral blood mononuclear leukocytes; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; TCGF, T cell growth factor; TLC, T lymphocyte clone.

of irradiated filler cells. In these studies, the following HA-specific clones were used, HA1.7 (specific for peptide 20) and HA2.61 (specific for peptide 11).

Proliferation Assays. TLC (2.5×10^4/ml) were cultured with HA (1.0 μg/ml) in the presence of irradiated autologous E^- cells (2.5×10^4/ml) in a total volume of 200 μl of supplemented RPMI 1640 containing 10% A^+ serum. In some experiments, as indicated, E^- cells were cultured for 18 h with antigen (1.0 μg/ml) and washed before the addition of cloned T cells. After 72 h incubation the cultures were pulsed for 8–12 h with 1.0 μCi of tritiated methyl thymidine ([^{3}H]TdR) (New England Nuclear, Boston, MA) and harvested onto glass fibre filters. Proliferation, as correlated with [^{3}H]TdR incorporation, was measured by liquid scintillation spectroscopy. The results are expressed as mean counts per minute ± standard error of the mean for triplicate cultures.

Culture Conditions for In Vitro Antibody Production. The methodology for the T cell-dependent production of specific anti-influenza antibody was based on that previously reported (24–27). TLC (HA1.7, 5×10^2) or E^+ cells (1×10^5) were cultured with autologous E^- cells (1×10^5) in round-bottomed 96-well microtiter trays (Linbro Chemical Co., Hamden, CT) in a total volume of 200 μl in RPMI 1640 containing 10% fetal calf serum (FCS) (Gibco Laboratories). To these cultures intact virus A/Texas or B/Sing (0.5 hemagglutinating units [HAU]/ml) were added. After 6 d incubation, the cells were washed and recultured in 100 μl of RPMI 1640 containing 5% FCS. Supernatants from triplicate cultures were collected at 24 h and stored at −20°C before assay for anti-influenza virus antibody.

Enzyme-linked Immunosorbent Assay (ELISA) for Anti-influenza Virus Antibody. Anti-influenza antibody was detected using an ELISA as described previously (25, 26). Flat-bottomed 96-well microtiter trays (Dynatech Laboratories Inc., Alexandria, VA) were coated with 100 μl of 500 HAU/ml of A/Texas or B/Sing in saline with 0.02% sodium azide for 1 h at 37°C in a humidified atmosphere. After blocking nonspecific binding sites with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) (Pentex Grade V; Miles Laboratories Inc., Elkhart, IN), 50 μl samples of culture supernatants were added to each well. After a further 1 h incubation, the trays were washed and to each well 100 μl of goat anti-human IgG conjugated to alkaline phosphatase (Miles Laboratories Inc.) diluted 1:1,000 in PBS-BSA was added. Before developing the reaction with 100 μl/ml of 1 mg/ml *p*-nitrophenyl phosphate (Sigma Chemical Co., St Louis, MO) in carbonate buffer (pH 9.8, 10^{-3} M $MgCl_2$) the tray was washed once with PBS-BSA and three times with the carbonate buffer. Absorbance was measured at 405 nm with a multichannel spectrophotometer (Dynatech Instruments Inc., Santa Monica, CA); from a standard curve of logit-transformed absorbance against log concentration of antibody of a reference serum, the actual amount of antibody was calculated (25). Backgrounds determined by replacing the test supernatant with RPMI 1640 containing 5% FCS were subtracted from the test values. The results are expressed as nanograms per milliliter.

Results

The Helper Activity of Clone HA1.7 in the Production of Anti-influenza Antibody. From previous studies, it was known that clone HA1.7 recognized peptide 20 of the HA1 molecule of the influenza Haemagglutinin, amino acid sequence 305–329 (28). The helper activity of HA1.7 was assayed on autologous E^- cells (a source of B cells and monocytes) in the presence of influenza A virus (A/Texas/1/77). The addition of 5×10^2 cloned T cells per culture was able to induce levels of specific antibody in excess of that induced by 10^5 unselected autologous E^+ cells (Table I). To determine the antigen specificity of the helper function, clone HA1.7 and autologous E^- cells were stimulated with A/Texas or B/Sing and then assayed for the production of anti-A/Texas or anti-B/Sing antibody (Table I). HA1.7 was able to induce anti-A/Texas antibody only when stimulated with A/Texas and not B/Sing. Furthermore, in the presence of B/Sing or A/Texas, HA1.7 was unable to cooperate with E^- cells in the production of B/Sing-specific antibodies (Table I). This could not be explained by the inability of the E^- cells to respond to B/Sing, since the addition of autologous E^+

TABLE I
*Helper Activity of Clone HA1.7**

Co-culture			Antibody response	
Helper cells	E^- cells	Antigen	Anti-A/Texas	Anti-B/Sing
			ng/ml	
E^+	+	A/Texas	147 ± 24	2 ± 1
+	−	+	0	0
−	+	+	2 ± 2	0
+	+	B/Sing	1 + 1	115 ± 33
+	−	+	0	0
−	+	+	0	5 ± 3
HA1.7	+	A/Texas	193 ± 17	0
+	−	+	0	0
+	+	B/Sing	0	4 ± 2
+	−	+	0	0

* Cloned helper T cells (HA1.7; 5×10^2) or E^+ (10^5) cells were cultured with autologous E^- (10^5) cells in the presence of A/Texas/1/77 or B/Singapore/222/79 (0.5 hemagglutinating U/ml). Anti-A/Texas and anti-B/Singapore antibody were determined in the supernatants of 7-d cultures. Background responses of TLC HA1.7, E^+, and E^- cells cultured alone with virus were measured.

cells leads to the production of anti-B/Sing antibody (Table I). Thus, the interaction of HA1.7 with autologous E^- cells is specific for A/Texas/1/77, as is the antibody synthesized.

Effect of Antigen Concentration on the Proliferative Response. The effect of antigen concentration on the proliferative response of TLC cells co-cultured with irradiated autologous E^- cells is shown in Fig. 1. The addition of HA-1 peptides 11 and 20 to clones HA2.61 and HA1.7, respectively, induced maximum proliferation over the concentration range of 0.3–3.0 μg/ml. The lower concentrations tested (0.01–0.3 μg/ml) were able to induce proliferation but at a suboptimal level. However, doses of peptide >10 μg/ml (10–300 μg/ml) diminished the response dramatically.

Antigen-induced Tolerance of TLC. To determine the cellular level at which high concentrations of antigen mediated their inhibitory effects, cells from HA1.7 were preincubated with differing concentrations of peptide 20 (0.01–300 μg/ml) in the absence of accessory cells. After 16 h incubation, the TLC were washed extensively and the viable cells (5×10^3) added to irradiated antigen-pulsed autologous E^- cells. Cloned helper cells (HA1.7) incubated in the presence of concentrations of peptide 20 >10 μg/ml were subsequently unable to respond when co-cultured with E^- cells pulsed with peptide 20 (Fig. 2). The effect was antigen specific in that preincubating HA1.7 cells with peptide 4 (300 μg/ml) did not inhibit the proliferative response of HA1.7 cells when added to E^- cells pulsed with peptide 20 (Fig. 2). Furthermore, after preincubation with any concentration of peptide 20 (over the range of 0.01–300 μg/ml), TLC were still fully capable of proliferating in the presence of TCGF, suggesting that the anergic state did not reflect a general inability of the cells to proliferate as a result of toxicity. To exclude the possibility that the unresponsiveness of the TLC was due to the carry over of irradiated T cells and/or accessory cells with suppressor function, TLC were cultured in the presence of TCGF alone for 7 d before pretreatment with antigen. It has been noted previously, using alloreactive TLC maintained on stimulator cells of unrelated HLA specificities, that after 7 d stimulator

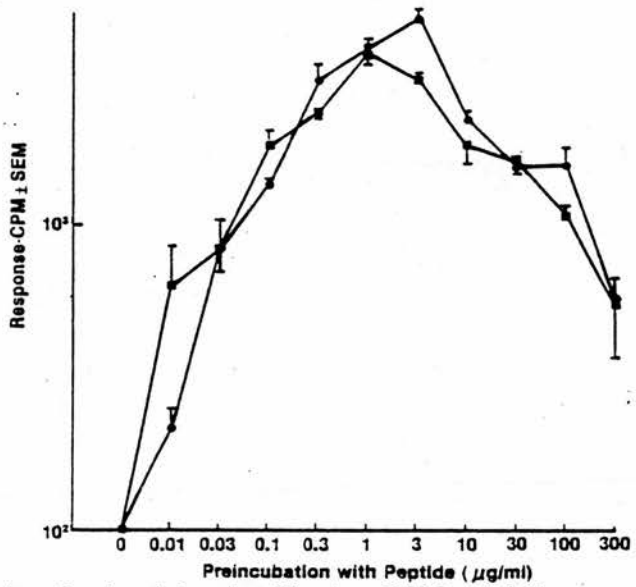

FIG. 1. Inactivation of antigen-induced proliferation of TLC with high concentration of specific antigen. TLC cells (HA1.7 [●], HA2.61 [■]; 5×10^3) were cultured with irradiated autologous E^- cells (5×10^3) in the presence of differing concentrations of antigen (0.01–300 μg/ml of peptides 20 and 11, respectively, for HA1.7 and HA2.61). Proliferation as correlated with [^{3}H]TdR incorporation was determined for 72-h cultures. The results are expressed as the mean counts per minute (cpm) ± SEM of triplicate cultures. Background responses of HA1.7 and HA2.61 in the absence of irradiated E^- cells for any of the antigen concentrations used was <50 cpm as was that of E^- cells alone cultured with antigen. The response of HA1.7 and HA2.61 cultured with E^- cells in the absence of antigen were 29 ± 4 and 21 ± 6 cpm, respectively.

cells were no longer detectable as determined by HLA phenotypic analysis (A. H. Johnson, Lombardi Cancer Research Center, personal communication). We have confirmed that in populations of irradiated filler cells cultured alone stimulator cells do not survive >7 d in vitro.

The duration of the exposure of helper T cells to antigen for tolerance induction was also investigated. TLC of HA1.7 were not rendered unresponsive during the first 60 min of incubation with specific antigen (peptide 20; Fig. 3). However, after 3 and 6 h pretreatment with antigen the ability of TLC to proliferate in the presence of antigen-pulsed E^- cells was reduced by 55 and 28%, respectively, and after 18 h the response was reduced to 4% of that seen with untreated T cells (Fig. 3). At each time point of preincubation with antigen, the response of the T cells when cultured in TCGF was not markedly different from that of the untreated population (Fig. 3). Although these results suggest that incubation with antigen for a duration of >3 h is required to induce unresponsiveness of >50%, cell density and the geometry of the wells in which the cells were cultured may influence the kinetics of tolerance induction, and these remain to be investigated in more detail.

The Influence of Accessory Cells on the Expression of Tolerance. It was considered that the number of accessory cells present in the assay culture may influence the expression of unresponsiveness in the pretreated T cells. However, variations in the number of accessory cells from 10^2 to 5×10^4 E^- cells per well did not appear to influence the state of unresponsiveness induced in the T cells of clone HA1.7 (Fig. 4). Furthermore,

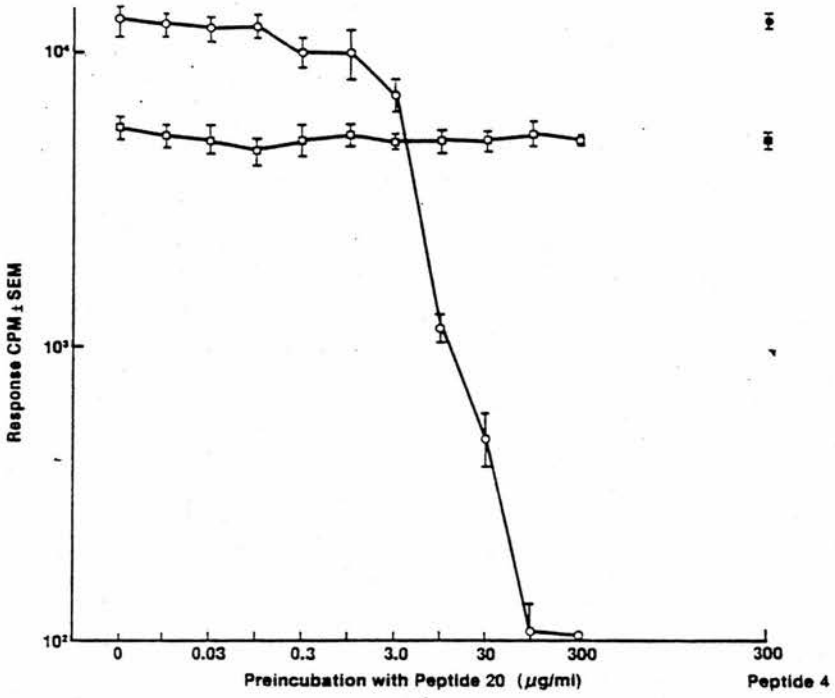

FIG. 2. Dose dependency of tolerance induced by preincubating T cells with specific antigen. Clone HA1.7 (10^5 cells/ml) was incubated in the presence or absence of varying concentrations of specific peptide (peptide 20; 0.01–300 μg/ml). The pretreatment was performed in round-bottomed 96-well microtiter plates for 16 h at 37°C. The plates were washed twice and 5×10^3 viable TLC cells were added to 5×10^3 irradiated antigen-pulsed E^- cells. Cells from each group were assayed for their ability to proliferate in the absence of TCGF alone. Proliferation was determined by [^{3}H]TdR incorporation as described in legend to Fig. 1. ○, HA1.7 preincubated with specific antigen (HA peptide 20) and then tested for the response to peptide 20 in the presence of accessory cells. □, HA1.7 preincubated with specific antigen (HA peptide 20) and then tested for the response to TCGF in the absence of accessory cells. ●, HA1.7 preincubated with non–cross-reactive antigen (HA peptide 4; 300 μg/ml) and then tested for the response to peptide 20 in the presence of accessory cells. ■, HA1.7 preincubated with non–cross-reactive antigen (HA peptide 4; 300 μg/ml) and then tested for the response to TCGF in the absence of accessory cells.

the unresponsiveness observed in the T cells pretreated with specific peptide could not be accounted for by a shift in the kinetics of the response since tolerized T cells co-cultured with varying numbers of irradiated E^- cells for 48, 72, and 96 h remained unresponsive (data not shown). In contrast, the initial rapid increase in the magnitude of the proliferative response of untreated cells of HA1.7 reached a plateau as the ratio of E^- cells to clone exceeded 1:1 (Fig. 4). Whether this plateau effect is due to inhibitory signals in the presenting cell population, or that the critical number of cells for maximal stimulation of the clone has been achieved, cannot be determined from these experiments, but it is clear that the usual 1:1 ratio used in the other experiments reported here is in the optimal range.

Antigen Specificity of Tolerance Induction. The antigen specificity of the induction of tolerance suggested in Fig. 2 was more fully analyzed using the combination of two T cell clones, HA1.7 and HA2.61, and the peptides 20 and 11 to which they are respectively specific. After preincubation with peptide (50 μg/ml), the cells were added to irradiated autologous E^- cells pulsed with peptide 20, 11, or both, and the

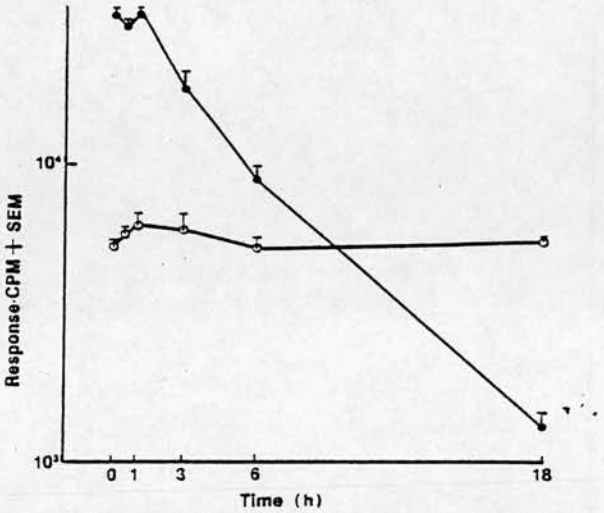

FIG. 3. TLC of HA1.7 (10^5/ml) were preincubated with 50 μg/ml of peptide 20 in the absence of E^- cells for 30 min, 1, 3, 6, and 18 h, then washed and assayed for proliferation as described in the legend to Fig. 2. ●, HA1.7 preincubated with HA peptide 20 and then tested for response on peptide 20-pulsed E^- cells. ○, HA1.7 preincubated with HA peptide 20 and then tested for response to TCGF in the absence of E^- cells.

proliferative response was determined. In addition, all experimental groups were evaluated for their ability to proliferate in the presence of TCGF alone. Clone HA1.7, specific for peptide 20, (group A, Table II) is rendered unresponsive by preincubation with peptide 20 (group B), but not by preincubation with the unrelated peptide 11 (group C). The reciprocal can be seen with HA2.61, which is specific for peptide 11 (group D), and can be tolerized by preincubation with peptide 11 (group E), but not with peptide 20 (group F). In contrast, all pretreated cells respond to TCGF to an extent identical to that of cells not pretreated with antigen (Table II).

In a more demanding test of specificity, the combination of the two test clones was incubated with peptide 11, 20, or both. The recovered cells were then assayed for their proliferative response on irradiated autologous E^- cells pulsed with peptide 11, 20 or both together. The data in Table III reveal that when the combination of clones HA1.7 and HA2.61 was preincubated with either peptide 11 or 20, the proliferative response was limited to peptide 20 or 11, respectively (groups B and C), compared with the normal response to each peptide observed in the group where the cells were preincubated in medium alone. However, when the cells were preincubated with both peptides, no response was seen when such cells were stimulated with accessory cells pulsed with 11, 20 or both peptides (group D). As before, a normal response to TCGF was seen in all groups regardless of the type of pretreatment experienced.

Duration of Tolerance. T helper cells of clone HA1.7 were tolerized with peptide 20 and then maintained in TCGF alone without the addition of filler cells for up to 96 h. The responses of these cells in the presence of antigen (peptide 20)-pulsed E^- cells or TCGF alone after various time in culture (16, 48, 72, 96, 168 h) were compared with those of untreated cells. The T cells remained unresponsive to stimulation with specific antigen in the presence of autologous E^- cells 168 h after being tolerized (Table IV). This unresponsiveness was not due to cytolytic effects since the T cells

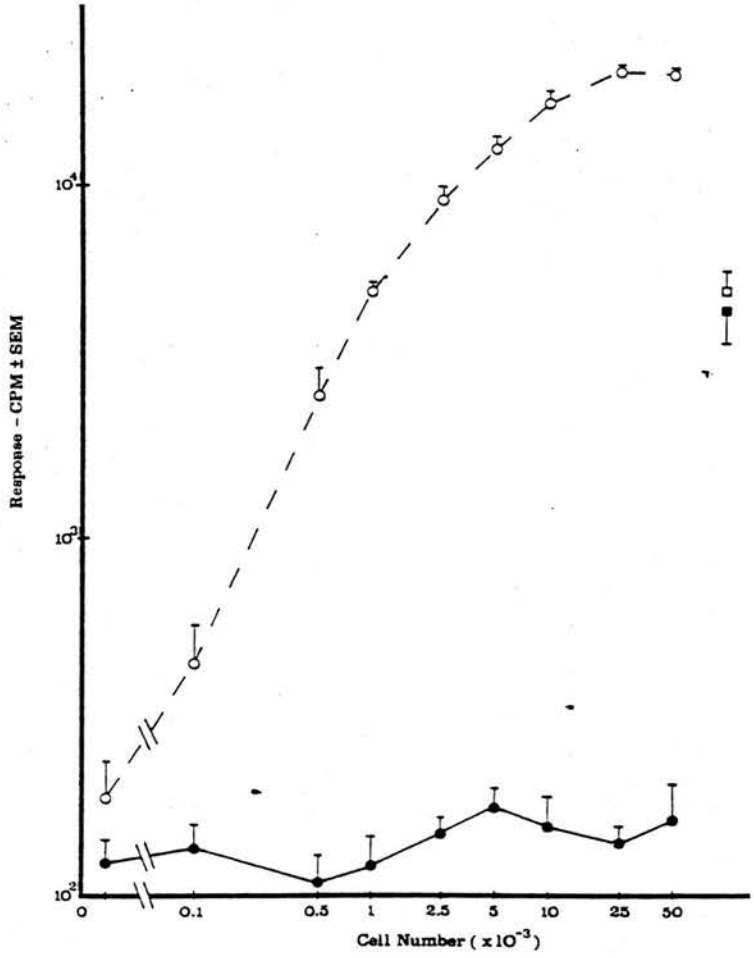

FIG. 4. The influence of accessory cell number on the expression of tolerance. Untreated and tolerized cells of HA1.7 (5×10^3/well) were assayed for responsiveness on irradiated antigen-pulsed E^- cells ranging in number from 10^2 to 5×10^4/well. The protocols for tolerance induction and the proliferative assay are described in the legend to Fig. 2. ●, HA1.7 preincubated with HA peptide 20 and then assayed for response on peptide 20-pulsed E^- cells. ○, untreated HA1.7 assayed for response on peptide 20-pulsed E^- cells. ■, HA1.7 preincubated with HA peptide 20 and then assayed for response to TCGF in the absence of E^- cells. □, untreated HA1.7 assayed for response to TCGF in the absence of E^- cells.

were able to proliferate in response to TCGF, and this response was not markedly different from that of untreated T cells maintained under the same culture conditions (Table IV). In contrast, the latter were able to respond to specific antigen (Table IV).

Discussion

There has been relatively little progress in the field of immunological tolerance in recent years. The reasons for this are not clear, but the lack of in vitro systems whereby the most significant form of tolerance, T cell tolerance, may be induced, has presumably contributed. The recent developments in cloning T cells has made it possible to envisage an in vitro model of tolerance induction, which may make it possible to discriminate between various possible mechanisms of tolerance induction as reviewed

TABLE II

*Specificity of Tolerance Induced by the Preincubation of Individual TLC with Antigen**

Tolerance Induction			Response			
Group	Clone	Antigen	Antigen			TCGF
			11	20	11 + 20	
			cpm + SEM			
A	HA1.7	None	129 ± 31	19,373 ± 1,257	18,638 ± 998	5,591 ± 292
B	HA1.7	20	135 ± 30	897 ± 145	918 ± 229	5,424 ± 221
C	HA1.7	11	86 ± 25	16,902 ± 1,369	19,293 ± 923	5,163 ± 187
D	HA2.61	None	6,548 ± 848	105 ± 12	7,591 ± 505	2,540 ± 176
E	HA2.61	11	237 ± 68	398 ± 52	495 ± 50	2,609 ± 230
F	HA2.61	20	7,055 ± 236	216 ± 13	7,256 ± 753	2,368 ± 100

* Cells from clone HA1.7 (specific for peptide 20) or HA2.61 (specific for peptide 11) were incubated for 16 h with either peptide 11 or 20 (50 μg/ml). Recovered cells (5×10^3/well) were assayed for proliferation in the presence of irradiated autologous E^- cells (5×10^3/well) that had been pulsed with peptide 11, 20, or both in the absence of E^- cells but in the presence of TCGF. Background responses of E^- cells in the presence or absence of antigen are <80 cpm. Results are expressed as described in the legend to Fig. 1.

TABLE III

*Specificity of Tolerance Induced by the Preincubation of Two Different TLC Cells with Each of Their Specific Antigens**

Tolerance induction			Response			
Group	Clone	Antigen	Antigen			TCGF
			11	20	11 + 20	
			cpm ± SEM			
A	HA1.7 + HA2.61	None	7,342 ± 929	18,474 ± 1,424	29,201 ± 1,637	9,182 ± 214
B	+ +	11	182 ± 29	16,446 ± 1,124	17,671 ± 2,702	9,141 ± 520
C	+ +	20	6,386 ± 271	485 ± 56	7,386 ± 595	9,598 ± 404
D	+ +	11 + 20	351 ± 30	501 ± 89	379 ± 77	9,620 ± 618

* The protocol is identical to that described in the legend to Table II with the exception that combinations of cells from clones HA1.7 and H2.61 were incubated with antigens.

by Weigle, Howard, and Mitchison (30–32). Two groups of mechanisms may be envisaged. Either antigen at supraoptimal concentrations acts to initiate the pathways that generate suppressor cells (reviewed in references 33, 34), or antigen may act directly on effector T cells, to modulate in some way their capacity to respond to antigen.

Recently suppressor T cells were cloned from a mouse rendered tolerant to BSA (35). This coupled to the general similarities between the conditions for tolerance and suppressor cell induction (e.g., in high antigen dose, accessory cell requirement [36, 37]) suggests that suppressor cells are often responsible for immunological tolerance as proposed by several authors (13–16). However, these results do not exclude the existence of tolerance induced by antigen in the absence of suppressor cells (e.g., 17). We thus performed experiments to determine whether antigen administered by itself will modulate the responsiveness of a clone of helper T cells in vitro. The results indicated that it is possible in vitro to induce specific immunological tolerance in a helper clone.

To determine whether antigen in excess would regulate the antigen-specific prolif-

TABLE IV
*Duration of Tolerance**

Time (post-antigen treatment)	Response of Cloned T helper cells			
	Tolerized		Untreated	
	E^- + Peptide 20	TCGF	E^- + Peptide 20	TCGF
h	*cpm ± SEM*			
16	13 ± 7	3,960 ± 210	11,277 ± 1,275	4,082 ± 491
48	153 ± 13	3,512 ± 308	10,331 ± 1,151	3,783 ± 375
72	179 ± 25	2,835 ± 435	14,068 ± 1,378	3,406 ± 455
96	127 ± 21	3,271 ± 227	9,517 ± 524	4,584 ± 339
168	488 ± 34	2,912 ± 354	12,485 ± 358	3,522 ± 210

* Cells from clone HA1.7 pretreated with peptide 20 were assayed for proliferation on E^- cells pulsed with specific peptide or in the presence of TCGF alone as detailed in the legend to Fig. 2. T cells were maintained at a concentration of 5×10^5/ml in TCGF alone for 16, 48, 72, 96, and 168 h after tolerance induction before use in the proliferation assay. In the proliferation assay both pretreated and normal T cells were added at 5×10^4 viable cells/well. As a control untreated cells were maintained under the same culture conditions. Background responses of E^- cells in the presence or absence of antigen at any of the time points taken are <100 cpm. Results are expressed as described in the legend to Fig. 1.

erative response, a dose-response analysis was performed, with the antigen present for the entire duration of the culture period. Supraoptimal concentrations yielded reduced proliferative responses to their respective immunogens, peptide 20 for clone HA1.7 and peptide 11 for clone HA2.61 (Fig. 1). From the literature on mouse T cell clones, it is clear that some clones do not yield this type of dose-response curve (e.g., 38).

Since it was determined that supraoptimal concentrations of synthetic peptide yield greatly reduced responses, preincubation experiments as previously described for the analysis of B cell tolerance were performed (10, 11). Because the presence of accessory cells or macrophages has been shown to inhibit or reduce the degree of tolerance (36) and of suppressor cell induction (37) these experiments were performed with T cell clones 7 d after the last irradiated filler cells were added. From previous work it is known that 2,500 rad-irradiated filler cells cannot be detected in our culture conditions after 7 d (A. H. Johnson, Lombardi Cancer Research Center, unpublished data).

It is observed that concentrations >3 μg/ml of the appropriate synthetic peptide (20) inhibited the proliferation of clone HA1.7 (Fig. 2), which is an HA-specific helper T cell (Table I). These concentrations of antigen are comparable to those required to induce B cell tolerance, for example in the polymeric flagellin system (10, 11) where 10–100 μg/ml was needed. The time course of antigen pretreatment was investigated, using 50 μg/ml of peptide 20 in the absence of E^- cells. Inhibition was detectable within 3 h, appreciable (~50%) by 6 h, and virtually complete by 18 h. Thus inhibition of T cells by antigen took 3–6 h or more, a result comparable to the case with B cells in vitro (10, 11).

The key criterion of immunological tolerance is that it is antigen induced, antigen specific and not immediately reversible. This aspect was investigated using two clones derived from the same individual, HA1.7, a helper cell that recognizes peptide 20, and HA2.61, which recognizes peptide 11. The findings reported here indicate that these clones are inhibited by the appropriate peptide only (Table I), even if a mixture of cells is used (Table II). This establishes the antigen specificity of the effect, and also excludes the possibility that a nonspecific diffusible inhibitor is released by cells

(43, 44). This is because effector cell blockade requires highly polymeric antigen (43), in contrast to the small synthetic oligopeptide we are using to tolerize T cells, and because effector cell blockade is fully recoverable within 24 h, unlike the stability of complete tolerance for at least 7 d noted here. Moorhead (45) has reported that dinitrophenyl (DNP)-specific delayed hypersensitivity T cells are blocked by preexposure to free DNP lysine (45). This phenomenon is different from the one described here, in that blocking by DNP lysine occurred rapidly (1 h at 4°C), and reversed rapidly (1 h at 37°C), whereas the tolerance induced by peptide 20 was unaltered by 7-d incubation at 37°C before challenge in vitro, and took much longer to induce (~16 h for 99% inhibition).

By developing a system for T cell tolerance within a T cell clone, we have a system for analyzing whether the nature of 'off signals', such as tolerance, differ quantitatively or qualitatively from the 'on signals'. Particularly relevant here is the question as to whether the induction of tolerance is MHC restricted (discussed in references 46, 47), which can be analyzed in this system and is under investigation.

Summary

Antigen-specific human T cell clones specific for defined peptides of influenza A hemagglutinin were found to be rendered unresponsive by incubation with moderately high concentrations of antigen. This was the case whether the synthetic peptide antigen was present for the duration of the culture or the cloned T cells were preincubated with antigen for 3–18 h at 37°C, before stimulation with T-depleted irradiated sheep erythrocyte non–rosette-forming lymphocytes (E^-) pulsed with the optimal dose of peptide. Tolerance could not be overcome by culture with various numbers of E^- cells and antigen. The induction of unresponsiveness was antigen specific, since it depended upon incubation with the appropriate peptide recognized by that clone. In addition, the tolerant T cells remained unresponsive to stimulation with the specific peptide for at least 7 d after induction even though maintained in culture in the presence of T cell growth factor. This state of antigen-specific unresponsiveness is akin to immunological tolerance. Furthermore, the experiments reported here demonstrate that the helper T cell clone can be inhibited by the relevant peptide in the absence of any suppressor cells or their precursors. This suggests that antigen-induced unresponsiveness need not always depend on the presence of suppressor T cells. The induction of tolerance in T cell clones does not result in early T cell death, since cells that no longer proliferate in response to the specific antigen and accessory cells still proliferate in response to T cell growth factor.

We thank Dr. J. Woody for his support, Dr. R. G. Webster for the gift of purified hemagglutinin, Dr. A. H. Johnson for HLA typing, Drs. R. A. Lerner, M. C. Raff, and N. A. Mitchison for their helpful comments, and Mrs. P. Wells for preparing the manuscript.

Received for publication 16 November 1982 and in revised form 26 January 1983.

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exposed to high concentrations of antigen—a 'bystander' inhibition. This mechanism must be considered, since bystander help has been described (29). The antigen specificity of the antigen-induced unresponsiveness fulfills the traditional criteria of 'immunological tolerance' (30, 31). The duration of the unresponsiveness is under analysis, but we know it lasts at least 7 d so that the effect is not transient.

This study indicates that it is now possible to analyze T cell tolerance in vitro at the clonal level. This is an advance over previous work which could only be performed at the population level, in vivo and in vitro. Furthermore, by using a population of cloned helper cells some conclusions concerning the mechanism of tolerance induction in vitro may be made. Since the viable T cells were helper cells, and irradiated (2,500 rad) filler cells used 7 d previously survive <7 d, and since irradiation is known to abrogate T cell suppression (39, 40), it thus appears that suppressor T cells are not essential for this form of antigen-induced immune regulation.

Furthermore, variation in the number of antigen-presenting cells in the assay cultures did not modulate the unresponsiveness observed when the T cells of clone HA1.7 were pretreated with antigen. This suggests that the unresponsiveness could not be the result of inhibitory signals originating in the presenting cell population and that the mechanism of tolerance induction operates at the level of the T cell.

It was noteworthy that clones, incapable of responding to antigen and E^- cells, were still fully responsive to TCGF. This indicates that the viability of the cells was not affected by the antigen. It also suggests that multiple receptors such as those for TCGF, antigen, etc., may influence the pathway leading to cell division independently. In these experiments only one aspect of immune responsiveness, namely antigen-induced specific proliferation was assessed. Other functions of these clones such as helper activity may be regulated independently, and this aspect requires investigation.

Our findings to date cannot assess the relative effectiveness or biological relevance of direct versus indirect (suppressor cell mediated) antigen-induced immunoregulation. Because of the high concentrations of peptide antigen used in these studies, with 50 μg/ml of a 24-amino acid peptide representing a molarity of $\sim 10^{-5}$ M, it may be expected that relatively few antigens will reach the concentrations needed to abrogate the proliferative response of recently activated T cells, as represented by the T cell clones used in this study. However, it is known that lymphocytes at different stages of maturation vary significantly in their capacity to be regulated by antigen. Thus immature B cells were sensitive to very low concentrations of antigen in vitro, a process termed 'clonal abortion' or more recently 'clonal anergy' by Nossal, Pike, and Boyd (41, 42). It is thus possible that T cells, at an earlier stage of maturation than those used here may be much more sensitive to direct antigen-induced inhibition, and it is also conceivable that antigen may regulate cell growth as assessed here, and differentiated functions such as help, independently.

The current evidence does not enable us to make further conclusions as to the mechanisms of tolerance, but clearly this is a powerful model system for analyzing postulated mechanisms of tolerance at the molecular and cellular level, such as receptor blockade or receptor loss (31, 32). Rapid clonal deletion (cell death) would appear not to be relevant to this type of tolerance as the cells could respond to TCGF normally for at least 7 d after the onset of unresponsiveness. The phenomenon under analysis here is not a T cell equivalent of effector cell blockade, in which interaction of our antibody-forming cells with antigen results in inhibition of antibody secretion

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Induction of Anergy in Human T Helper 0 Cells by Stimulation with Altered T Cell Antigen Receptor Ligands[1]

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CD4+ T cells may become profoundly unresponsive to antigenic restimulation following ligation of TCR by immunogenic peptides bound to MHC class II molecules in the absence of costimulation. Furthermore, it has been reported that anergy can be induced as a consequence of engagement of TCR by analogues of antigenic peptides presented by live APCs. In this study, based on resolution of the crystal structure of an influenza virus hemagglutinin (HA) peptide (HA 306–318) bound to HLA-DRB1*0101, we investigated the potential of analogues with amino acid substitutions at those positions predicted to form interactions with TCR to differentially activate and/or anergize HA-specific human Th0 cells restricted by DR1 class II molecules. For some analogues altering the affinity of peptide/TCR interactions revealed a direct positive correlation between antigenicity and their ability to induce anergy. Nevertheless, certain HA peptide analogues functioned as partial agonists, which although they failed to stimulate clonal expansion, were capable of rendering the Th0 cells unresponsive to immunogenic rechallenge. Furthermore, differences were noticed in the characteristics of the anergic phenotype induced by selected analogues. Restimulation with the native peptide of Th0 cells pre-exposed to the HA analogues in the absence of costimulatory signals failed to uncouple IL-4 and IFN-γ secretion; however, in some instances, dissociation of proliferation from cytokine production was observed. The ability to differentially signal T cells through changing the affinity of peptide/TCR interactions may have implications in the potential use of altered TCR ligands in immunotherapy. *The Journal of Immunology*, 1996, 156: 2801–2808.

Engagement of TCR by antigenic peptides bound to class II molecules encoded by the MHC, together with costimulatory signals provided by APC, initiate the effector functions of CD4+ T cells (e.g., 1–4). In contrast, altered signaling of T cells by ligation of the TCR, in the absence of costimulation, leads to a state of functional unresponsiveness termed anergy, which is characterized by a failure of the T cells to proliferate when restimulated (e.g., 4–9). From the analysis of in vitro systems, using either human or murine CD4+ T cells, it has been reported that anergy may be achieved by stimulation with native peptide under the appropriate conditions. For example, human CD4+ T cells when exposed to supraimmunogenic concentrations of peptide, in the presence or absence of APCs, become anergic to immunogenic restimulation (5), whereas for murine T cells unresponsiveness can be induced by stimulation with antigenic concentrations of native peptide presented by chemically modified APCs (e.g., Refs. 6, 10). The early studies on murine T cell anergy focused on the Th1 functional phenotype and established that impaired IL-2 transcription in the anergic T cells resulted in the loss of Ag-dependent proliferation (5). From the analysis of the kinetics of cytokine-specific mRNA induction it has been demonstrated that human T cells become hyperactivated during the initial exposure to tolerizing concentrations of peptide (11); however, when the anergic human T cells were restimulated, a defect in IL-2 production was observed (12).

Attempts to induce anergy in murine Th2 cells by ligation of the TCR with native peptide in the absence of costimulation, in general, have been unsuccessful (4, 13, 14). However, recently the ability of peptide analogues with partial agonist effects to modulate the effector functions of both murine Th1 and Th2 cells has been studied (9, 10, 15, 16). These analogues of immunogenic peptides, termed altered peptide ligands, bind with comparable affinity to class II MHC molecules but have altered affinities for TCR. The presentation of altered peptide ligands by live APCs can induce murine Th2 (10) cells, as well as Th1 (9) cells, to become profoundly unresponsive to restimulation with wild-type peptide, suggesting that the regulation of clonal expansion is fundamental to the induction of anergy in both Th1 and Th2 cells. However, the Th2 cells retained the capacity to secrete IL-4, which raises the possibility that the presence of IL-4 may confer resistance to the induction of complete anergy in Th2 and Th0 cells or that transcriptional regulation of IL-4 is not subject to modulation during anergy induction (7, 10).

These observations prompted us to investigate the potential to anergize human Th0 cells, as well as to inhibit or dissociate the production of Th1 (IFN-γ)- and Th2 (IL-4)-derived cytokines on restimulation of native peptide by using altered peptide ligands with the properties of agonists or partial agonists. The resolution of the crystal structure of hemagglutinin (HA)[3] 306–318/DRB1*0101 complexes (17, 18) and the interaction of the native HA peptide and analogues with affinity purified and membrane-expressed DRB1*0101 molecules in binding studies (19, 20) imply that residues at positions 307, 309, 310, 312, 315, and 318 interact with the TCR. Based on this prediction we have generated a panel of peptide analogues with amino

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Received for publication October 2, 1995. Accepted for publication January 29, 1996.

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[1] This work was supported by grants from the Wellcome Trust, U.K., the Medical Research Council, U.K., and the Onassis Foundation (DT).

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[3] Abbreviation used in this paper: HA, hemagglutinin.

0022-1767/96/$02.00

acid substitutions at these positions and investigated their effects on the induction of anergy in cloned human Th0 cells of the appropriate Ag and restriction specificity (21).

Materials and Methods

Antigens

The influenza virus HA peptide residues 306–318 (PKYVKQNTLKLAT) and analogues with substitutions at the TCR contact residues were synthesized on a Multipeptide Synthesis block BT7400 (Cambridge Research Biochemicals, Northwich, U.K.) using 4-(2', 4' dimethoxyphenyl F-moc aminomethyl)phenoxy resins (Novasyn Chemicals, Nottingham, UK) as previously described (22). Couplings were performed using F-moc side chain-protected pentafluorophenyl or oxobenzotetiazine amino acid esters. Full-length peptides were cleaved from the resin and deblocked by treatment with trifluoroacetic acid-thioanisole-ethanedithiol (94:5:2.5, v/v) for 1 h and then precipitated and washed twice with ether. The precipitates were dissolved in 10% formic acid (v/v) and freeze dried for 48 h. Each peptide produced a single peak by reverse-phase HPLC. The peptide analogues used in this study are listed later in Table I.

Cell surface binding assays

The binding affinities of native and peptide analogues of HA 306–318 were compared by their ability to compete with biotinylated HA 306–318 for binding to DRB1*0101 expressed on murine fibroblasts as previously described (19). DRB1*0101-positive fibroblasts were co-incubated with NH-long chain biotinylated HA peptide (25 μg/ml) and selected analogues (500 μg/ml), washed, and incubated with fluoresceinated avidin D (Vector Laboratories, Peterborough, U.K.) followed by biotinylated anti-avidin D. The cells were then incubated again with fluoresceinated avidin D and fluorescence intensity was determined by flow cytometry.

*Isolation of HA 306–318 reactive DRB1*0101-restricted cloned human Th0 cells*

Cloned T cells of HA1.7 were isolated by limiting dilution cloning method as described previously (21). The T cell clone was maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 IU/ml penicillin/streptomycin (Life Technologies, Paisley, Scotland) and 5% inactivated human A^+ serum (National Blood Transfusion Service, Edgware, U.K.) by stimulating with HA 306–318 peptide and irradiated histocompatible PBMC (5×10^5/ml; 3000 rad) as a source of APCs together with IL-2 (10% v/v; Lymphocult T, Biotest Folex, Frankfurt, Germany). In all experiments, clones were tested 7 to 8 days after the last addition of Ag and APCs.

Proliferation assays

Cloned T cells (HA1.7; 2.0×10^4/well) were cultured in flat-bottom, 96-well microtiter trays (Nunc, Roskilde, Denmark, and Life Technologies) with analogues of HA 306–318 (0.003 to 100 μg/ml) in the presence of APCs. Mitomycin C-treated murine fibroblasts expressing HLA-DRB1*0101 (2×10^4 cells/well), irradiated (5000 rad) homozygous DRB1*0101 expressing EBV-transformed B cells lines (2×10^4 cells/well), or irradiated (3000 rad) histocompatible PBMCs were used for Ag presentation. From selected cultures, supernatants were collected at 48 h and the levels of IL-4 determined. Cultures were pulsed with [^{3}H]TdR (1 μCi/well) (Amersham, Amersham, U.K.) after 48 h and harvested 8 to 16 h later. Proliferation was determined by [^{3}H]TdR incorporation by liquid scintillation spectroscopy. The results are expressed as mean cpm for triplicate cultures with SEM <20%.

Induction of T cell anergy

T cells (10^6/ml) were cultured with peptide (100 μg/ml) or in medium alone or in the presence of APCs for 18 h and then washed extensively before being rechallenged with either an optimal concentration of native peptide in the presence of APCs (DR1-expressing murine fibroblasts) or IL-2 alone as described previously (5). Proliferation was measured by the incorporation of [^{3}H]TdR.

Measurement of IFN-γ and IL-4

Supernatants were collected at 48 h from cultures of peptide-pretreated or untreated T cells rechallenged with immunogenic peptide. Triplicate wells were pooled and IFN-γ and IL-4 were measured by ELISA as described previously (23).

Cytofluorometric analysis

T cells were stained for cell-surface phenotype by standard methods using mAbs (CD2, CD3, and CD25) directly conjugated to FITC and analyzed on an EPICS IV (Coulter, Hialeah, FL) (24).

Results

Effect of amino acid substitutions at the TCR contact residues on the ability of the HA 306–318 peptide to stimulate proliferation, induce anergy, or modulate cytokine (IL-4 and IFN-γ) production by anergic T cells

Peptide analogues of HA 306–318, with amino acid substitutions at positions 307, 309, 310, 312, and 315, but not 318, since the threonine at this position is not required for recognition by HA1.7 cells (25), were investigated for agonist effects. The MHC class II-binding characteristics of the HA peptide analogues used in this study have been investigated in detail by others (19, 20). However, to confirm their previous observations that substitutions at these positions had minimal effects on MHC class II-binding, selected analogues were investigated in cell surface binding assays. The HA analogues tested with substitutions at positions 307, 309, 310, 312, and 315 (Table I) inhibited the binding of biotinylated native peptide to a similar degree as the unlabeled native HA 306–318 (Fig. 1). As an additional control, binding was determined for an analogue in which tyrosine at position 308, a residue critical for MHC class II binding (17–20), had been substituted by serine (S^{308}). The binding of labeled peptide was only weakly inhibited by S^{308} suggesting that it has a lower affinity for DRB1*0101 than either native peptide or the other analogues tested.

Substitutions at position 307. Compared with the native peptide, which has a lysine at position 307 (K^{307}), the magnitude and shape of the dose-response curve of proliferation induced by the analogue with a conservative substitution (R^{307}) was similar. Over the concentration range of 3 to 30 μg/ml, the analogues E^{307}, G^{307}, H^{307}, and Q^{307} stimulated proliferation at reduced levels compared with the native peptide. However, at 30 μg/ml proliferation induced by H^{307} and Q^{307} was greater than that of K^{307} (Fig. 2*A*).

Since it has been reported that altered T cell ligands are able to dissociate proliferation from IL-4 production (15), the supernatants from cultures in which the analogues induced only minimal or no proliferation were analyzed for the presence of IL-4 (Fig. 3). The analogues E^{307} and G^{307}, which stimulated reduced proliferation, also induced only low levels of IL-4 compared with native peptide. Only the levels of IL-4 induced by peptide at concentrations that stimulate maximum proliferation are presented.

In order to determine whether the analogues were able to induce anergy, T cells were cultured with each peptide (100 μg/ml) in the absence of APCs and both proliferation and cytokine production measured on restimulation by APCs pulsed with an antigenic concentration of native peptide. HA1.7 cells failed to proliferate (Fig. 2*B*) or produce IL-4 and IFN-γ (Fig. 2*C*) on restimulation after incubation with the native peptide or the analogues R^{307}, G^{307}, and H^{307} as compared with the control of cells maintained in medium alone. HA-E^{307} partially inhibited Ag-induced proliferation, but had less of an effect on cytokine production. In contrast, Q^{307} failed to induce anergy. As previously described for the native HA, peptide responsiveness of anergic T cells to exogenous IL-2 was enhanced (24). This effect was also observed for the HA^{307} analogues, which induced anergy, with the exception of G^{307} in which no enhancement of IL-2 responsiveness was observed (Fig. 2*B*).

Substitutions at position 309. When valine (V^{309}) in the native peptide was substituted by phenylalanine (F^{309}) or lysine (K^{309}), minimal or no proliferation was observed (Fig. 4*A*). Neither did these analogues induce IL-4 production (Fig. 3). The shape of the

Table I. *Amino acid sequences of native peptide and analogues of HA 306–318*

	307			309	310			312			315			
P	K	Y		V	K	Q		N	T	L	K	L	A	T
	E													
	G													
	H													
	Q													
	R													
				A										
				F										
				G										
				H										
				K										
					A									
					D									
					E									
					G									
					R									
								A						
								E						
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											V			

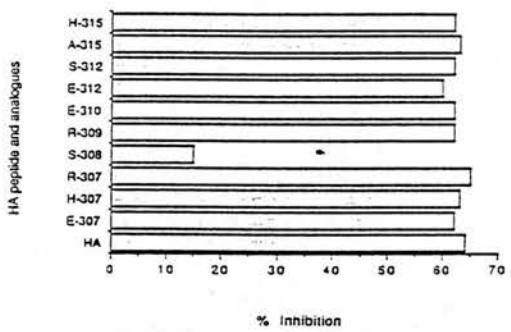

FIGURE 1. Binding of native HA 306–318 peptide and analogues to DRB1*0101. DRB1*0101 transfectants were cocultured with unlabeled native peptide and analogues together with biotinylated HA 306–318 and the level of binding was analyzed by flow cytometry.

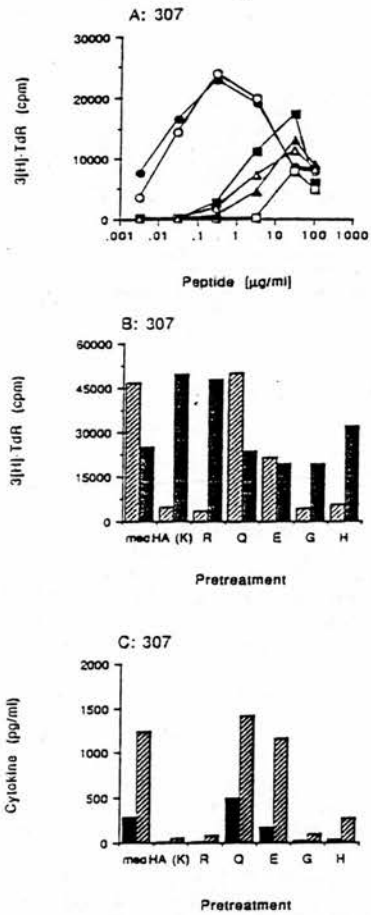

FIGURE 2. Effects of amino acid substitutions at position 307 in HA 306–318 on T cell recognition and the induction of anergy. *A*, Cloned T cells were stimulated with the following HA^{307} analogues: ○, R^{307}; ■, Q^{307}; □, E^{307}; ▲, G^{307}; △, H^{307}; and native peptide (HA-K^{307}), ● in the presence of APCs (mitomycin C-treated HLA-DRB1*0101 expressing murine fibroblasts). [^{3}H]TdR incorporation was determined at 72 h. *B*, HA1.7 cells were stimulated with native peptide (HA-K^{307}), R^{307}, Q^{307}, E^{307}, G^{307}, and H^{307} in the absence of APCs and then restimulated with APCs and native peptide (cross-hatched bars) or IL-2 alone (stippled bars), and proliferation was determined by the incorporation of [^{3}H]TdR. *C*, Cloned T cells were stimulated with native peptide (HA-K^{307}), R^{307}, Q^{307}, E^{307}, G^{307}, and H^{307} in the absence of APCs and restimulated with APCs and native peptide, and IL-4 (solid bars) and IFN-γ (cross-hatched bars) were measured in supernatants collected at 48 h.

dose-response curves of the proliferation induced by the substitutions A^{309}, G^{309}, and H^{309} was similar to that of V^{309}, but reduced in magnitude with H^{309}, least potent of the analogues (Fig. 4*A*).

Pretreatment with F^{309} or K^{309} failed to reduce either proliferation (Fig. 4*B*) or cytokine production (Fig. 4*C*) on restimulation. In contrast, these T cell functions were down-regulated by the A^{309}, G^{309}, and H^{309}. Although H^{309} was markedly less antigenic than native peptide, it had similar potency in terms of inducing anergy. Enhanced IL-2 responsiveness paralleled loss of Ag-dependent proliferation (Fig. 4*B*).

Substitutions at position 310. The capacity of HA^{310} analogues to induce anergy paralleled their antigenicity. Of the analogues screened (A^{310}, D^{310}, E^{310}, G^{310}, and R^{310}) only the conservative substitution of arginine for lysine stimulated proliferation and IL-4 production, nevertheless, this was reduced in the order of two logs (Fig. 5*A*). Pretreatment with R^{310} induced anergy, inhibited proliferation (Fig. 5*B*) and cytokine production (Fig. 5*C*), and enhanced responsiveness to exogenous IL-2.

Substitutions at position 312. In the native peptide, asparagine (N^{312}) was found at position 312. The analogues A^{312} and S^{312} were antigenic but reduced compared with N^{312} (Fig. 6*A*). Stimulation with S^{312} also induced low levels of IL-4 production as

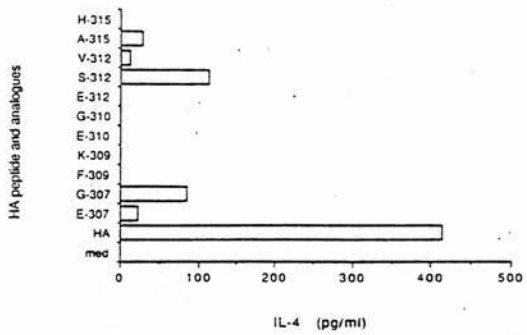

FIGURE 3. IL-4 production by T cells stimulated with HA analogues in the presence of APCs. T cells of HA1.7 were cultured with the native peptide and analogues in the presence of APCs, and the level of IL-4 was measured in 48-h supernatants.

compared with the native peptide (Fig. 3). E^{312}, V^{312}, and the conservative substitution Q^{312} all failed to induce proliferation.

A^{312}, similar to N^{312}, induced anergy (Fig. 6*B*) and inhibited cytokine production (Fig. 6*C*) on restimulation, in contrast to Q^{312}. Pretreatment with S^{312} induced modulation of IL-4 and IFN-γ production but failed to inhibit the proliferative response on restimulation. E^{312} failed to stimulate proliferation but, nevertheless, induced partial anergy of Ag-dependent proliferation and inhibited both IL-4 and IFN-γ production without increasing responsiveness to IL-2. Stimulation with V^{312} resulted in down-regulation of cytokine production but Ag-dependent proliferation was only partially anergized and no increase in IL-2 responsiveness was observed (Fig. 6, *B* and *C*).

Substitutions at position 315. None of the analogues substituted at position 315 was as efficient as the native peptide (K^{315}) in stimulating proliferation of HA1.7 cells (Fig. 7*A*). The conservative substitution, R^{315}, was antigenic at 0.3 μg/ml and greater but was three logs less potent. A^{315}, V^{315}, and D^{315} induced limited proliferation, whereas H^{315} failed to stimulate the T cells. Stimulation with H^{315} failed to induce IL-4 production, although A^{315} was able to stimulate suboptimal levels (Fig. 3).

After pretreatment with A^{315}, V^{315}, and D^{315}, the T cells responded by proliferation (Fig. 7*B*) and cytokine release (Fig. 7*C*) when rechallenged with the native peptide. R^{315}, similar to K^{315}, anergized the proliferative response and both IL-4 and IFN-γ production. H^{315}, which failed to stimulate HA1.7 cells, induced complete anergy (Fig. 7, *B* and *C*). The induction of unresponsiveness by H^{315} was dose dependent (Fig. 8*A*). However, unlike K^{315} and R^{315}, exposure to H^{315} did not enhance responsiveness to exogenous IL-2. Pretreatment of the T cells with H^{315} or native peptide in the presence of APCs resulted in the induction of T cell anergy (Table II).

Increasing the concentration of native peptide used to rechallenge anergic T cells failed to stimulate clonal expansion or cytokine production. Results are presented for the proliferative response of HA1.7 cells anergized by native peptide or the analogues G^{307}, E^{312}, or H^{315} (Fig. 8*B*). Furthermore, stimulation of HA1.7 cells in the presence of different types of APCs (DRB1*0101 expressing murine fibroblasts, EBV-transformed B cells, or PBMC) did not alter the pattern of response in the proliferation assays. Likewise, the degree and characteristics of anergy induced by native peptide or HA analogues were not affected by APCs present at the time of restimulation (data not shown).

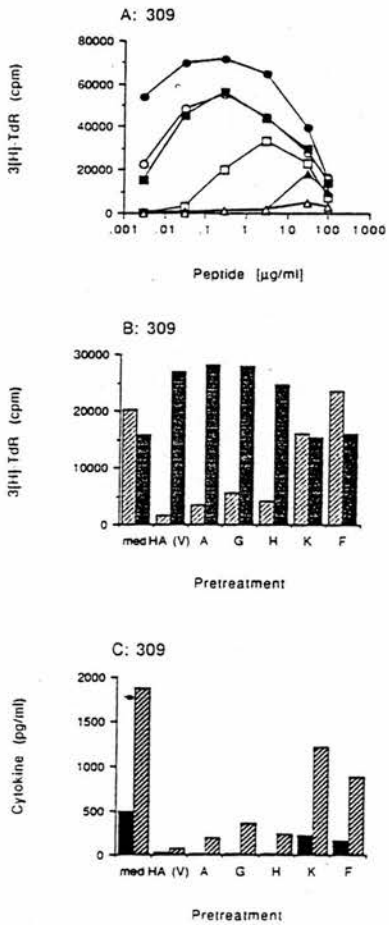

FIGURE 4. The effects of amino acid substitutions at position 309 in HA 306–318 on T cell recognition and the induction of anergy. The following HA^{309} analogues: ○, A^{309}; ■, G^{309}; □, H^{309}; ▲, K^{309}; △, F^{309}; and the native peptide ($HA\text{-}V^{309}$) (●) were investigated. The results are presented as described in the legend to Figure 1.

Phenotypic modulation of anergic Th0 cells

Peptide-mediated anergy modulates the cell surface phenotype of human $CD4^+$ T cells, and as previously reported (24), the expression of the TCR/CD3 receptor complex was down-regulated while membrane levels of CD2 and CD25 were elevated (Fig. 9). The effect of preincubation of the T cells with selected HA analogues of HA 306–318 was also analyzed. The HA-307 analogues (G^{307}, H^{307}, and R^{307}), which induced anergy, also down-regulated CD3 expression. However, although membrane levels of both CD2 and CD25 were enhanced by R^{307}, expression of CD2 but not CD25 was elevated following stimulation with G^{307}. Only minimal up-regulation of CD25 was observed following incubation with H^{307} (Fig. 9). The partial anergy induced by E^{312} was accompanied by marginal reduction in CD3 expression and enhancement of CD25, with CD2 unaltered. In contrast, Q^{312} enhanced CD2 and CD25 cell surface levels, but had limited effect on CD3. As regards the HA-315 analogues, for D^{315} the phenotypic modulation paralleled

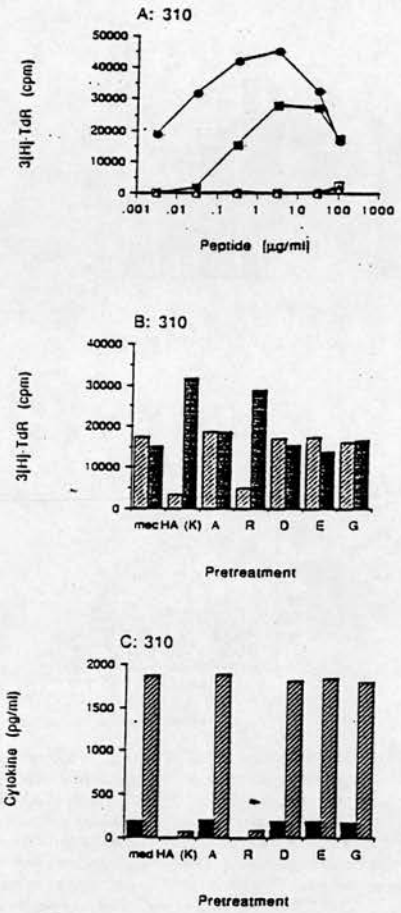

FIGURE 5. Effects of amino acid substitutions at position 310 in HA 306–318 on T cell recognition and the induction of anergy. The following HA^{310} analogues: ○, A^{310}; ■, R^{310}; □, D^{310}; ▲, E^{310}; △, F^{310}; and the native peptide (HA-K^{310}) (●) were investigated. The results are presented as described in the legend to Figure 1.

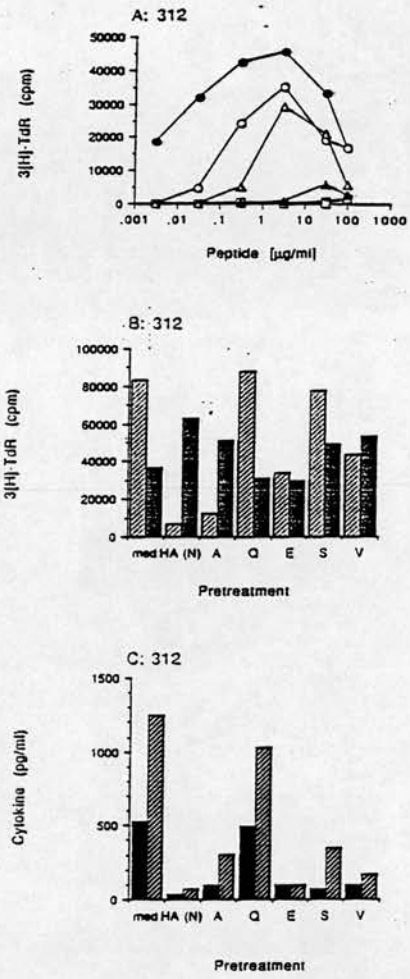

FIGURE 6. Effects of amino acid substitutions at position 312 in HA 306–318 on T cell recognition and the induction of anergy. The following HA^{312} analogues: ○, A^{312}; ■, Q^{312}; □, E^{312}; ▲, V^{312}; △, S^{312}; and the native peptide (HA-N^{312}) (●) were investigated. The results are presented as described in the legend to Figure 1.

functional responses to Ag rechallenge or exogenous IL-2. H^{315}, which induced anergy, partially down-regulated CD3, up-regulated CD2, but had little effect on CD25 membrane expression, which was in agreement with the lack of enhanced responsiveness to exogenous IL-2. A similar pattern of phenotypic modulation was observed when the T cells were exposed to anergizing concentrations of H^{315} or native peptide with APCs present in the primary cultures.

Discussion

In this report we have investigated the ability of peptide analogues of HA 306-318 with altered affinities for the TCR to induce proliferation and anergy in human HA-specific Th0 cells. For the majority of analogues we have demonstrated a direct positive correlation between immunogenicity and their ability to induce anergy. However, for selected peptides, such as Q^{307}, E^{312}, and H^{315}, we observed that clonal expansion could be dissociated from the induction of anergy (Table III). We have previously reported that stimulation of human $CD4^+$ T cells with supraimmunogenic concentrations of wild-type peptide, in the absence of costimulatory signals, can render the cells unresponsive to further immunogenic challenge (5, 24). Here we extended those findings and demonstrated that exposure of Th0 cells to certain HA analogues (e.g., S^{312}), under similar conditions, may modulate their effector activity as regards their capacity to proliferate and secrete cytokines when subjected to immunogenic restimulation.

Analyzing the patterns of proliferation of HA1.7 cells, in the presence of APCs, revealed that lysine at position 310 in the HA peptide was critical for Ag recognition since substitutions of this residue, including a conservative change to arginine, were poorly tolerated. It would appear, therefore, that position 310 is a primary

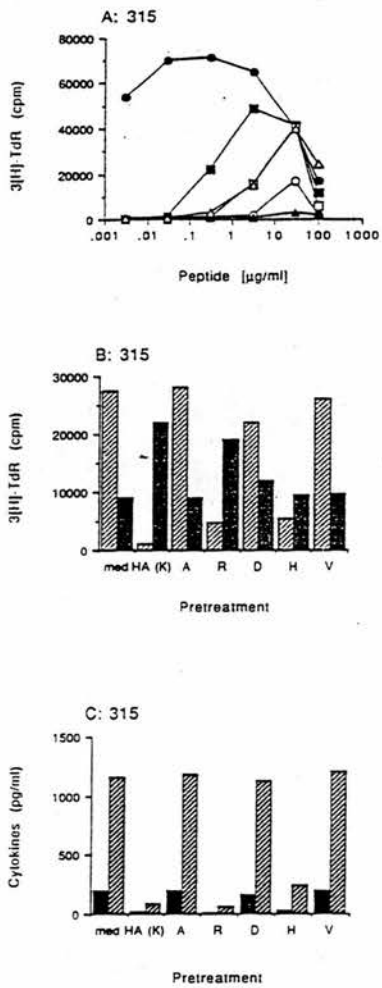

FIGURE 7. Effects of amino acid substitutions at position 315 in HA 306–318 on T cell recognition and the induction of anergy. The following HA^{315} analogues: ○, A^{315}; ■, R^{315}; □, D^{315}; ▲, H^{315}; △, V^{315}; and the native peptide (HA-K^{315}) (●) were investigated. The results are presented as described in the legend to Figure 1.

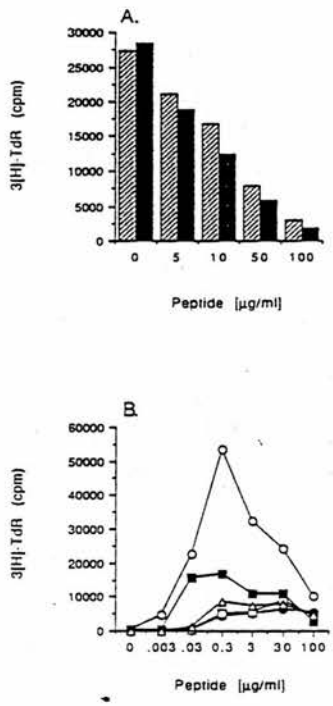

FIGURE 8. Effect of Ag concentration on anergy induction and restimulation. *A*, Dose response curve of H^{315}-induced T cell anergy. HA1.7 cells were cultured in medium or with increasing concentrations of H^{315} (cross-hatched bars) or native (solid bars) peptide in the absence of APCs. T cells were restimulated with APCs and native peptide and proliferation measured. *B*, Effect of Ag concentration on the restimulation of anergic T cells. HA1.7 cells were cultured in medium (○) or with C^{307} (□); E^{312} (■); H^{315} (△), or the native peptide (●) at 100 μg/ml in the absence of APCs and then restimulated with increasing concentrations of native peptide in the presence of APCs. Proliferation was determined by the incorporation of [^{3}H]TdR.

Table II. *Induction of T cell anergy in the presence of APCs*

Peptide Pretreatment[a]	Ag Rechallenge HA 306 + APC	Surface Phenotype	
		CD3	CD25
Medium	19,350	55	13
HA	1,876	38	315
H-315	2,012	41	100

[a] T cells were cultured with APCs (DRB1*0101 expressing murine fibroblasts) alone or in the presence of native peptide or H^{315} (100 μg/ml) for 38 h. Phenotypic expression of CD3 and CD25 was determined and the proliferative response of the T cells to restimulation with native peptide in the presence of APCs assessed as described in the legend to Figure 1.

TCR contact residue (26). In contrast, the residues at positions 307, 309, 312, and 315 have the characteristics of secondary TCR contact residues, as a variety of changes in the nature of the amino acids at these positions were accepted and this confirmed our earlier observations on the contribution of positions 309 and 312 to Ag recognition by HA1.7 cells (27). In general, peptide/MHC class II complexes formed with the HA analogues substituted at either the primary or secondary TCR contact sites revealed a direct correlation between immunogenicity, both in terms of proliferation and IL-4 production and their ability to induce anergy. Similar observations have been reported for other in vivo experimental systems (e.g., 28, 29). The capacity of amino acid substitutions at secondary TCR contacts to modulate selected effector functions has been studied in most detail for murine T cell clones specific for hemaglobin peptide Hbβ^d (64–76) (9, 10, 15, 16). It has been demonstrated that one analogue of this peptide with a conservative substitution (S→A at position 70), although unable to stimulate Th1 cells either to proliferate or produce cytokines, when presented by live APCs was able to render them unresponsive to challenge with wild-type peptide (9). Similarly, we observed that stimulation of HA1.7 cells with selected analogues, in which the secondary TCR contacts had been substituted (e.g., E^{307}, G^{307},

Table III. *Summary of effects of altered T cell ligands on activation and induction of anergy*

	Activation[a]		Peptide Pretreatment[b]	Proliferation of T Cell Anergy		Cytokines	
	Proliferation	IL-4		HA + APC[c]	IL-2	IL-4	IFN-γ
Medium	−	−	Medium	+++++	++	+++++	+++++
HA	+++++	+++++	HA	−	−+++++	−	−
E-307	+	+/−	E-307	++	−−	++++	++++
G-307	++	+/−	G-307	−	−+	−	−
E-312	−	−	E-312	++	+	−	−
S-312	++	++	S-312	++++	++	−	+
V-312	+/−	+/−	V-312	+++	−−+	+/−	+/−
H-315	−	−	H-315	+/−	−+	−	−

[a] T cells were cultured in the presence of APCs (DRB1*0101 transfectants) in the presence and absence of native peptide and analogues of HA 306–318.
[b] T cells were pretreated with peptides in the absence of APCs.
[c] Peptide-pretreated T cells were rechallenged with native HA 306 in the presence of APCs.

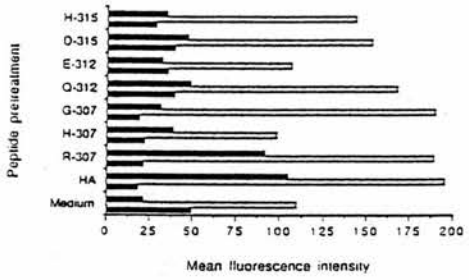

FIGURE 9. Phenotypic modulation of HA1.7 cells stimulated with HA analogues in the absence of costimulation. HA1.7 cells were cultured in medium, R^{307}, H^{307}, G^{307}, Q^{312}, E^{312}, D^{315}, H^{315}, and the native peptide all at 100 μg/ml in the absence of APCs and cell-surface expression of CD2 (cross-hatched bars), CD3 (solid bars), and CD25 (stippled bars) measured by fluorescence.

E^{312}, V^{312}, S^{312}, H^{315}) interfered with qualitative and quantitative aspects of proliferation and anergy induction in the presence and absence of APCs.

Peptide-mediated anergy of HA1.7 cells was characterized by modulation of the cell-surface phenotype with reduced expression of TCR-CD3 receptor complexes and enhanced membrane levels of CD2 and CD25 (24, 30). Nevertheless, different patterns of modulation were observed following exposure to selected analogues; for example, anergy induced by G^{307} had minimal effect on cell-surface expression of CD25. Therefore, changes in the affinity of TCR/ligand interactions appeared to affect not only the capacity to stimulate clonal expansion or induce anergy but also qualitative differences in these functional outcomes. This was further illustrated by analysis of the activity elicited by certain HA peptides with substitutions at positions 312 and 315. In the presence of APCs, stimulation with S^{312} induces proliferation, albeit reduced in magnitude when compared with the native peptide. Nevertheless, this analogue induces partial anergy affecting only cytokine production on restimulation. Without costimulation exposure to E^{312} or V^{312}, nonimmunogenic analogues resulted in inhibition of both IFN-γ and IL-4 production on rechallenge, whereas the proliferative response was only partially inhibited. The H^{315} peptide analogue appeared to form partial agonist complexes with DR1 that were able to inhibit both cytokine production and proliferation, although in the presence of APCs they failed to induce T cell proliferation. The induction of anergy by H^{315} and E^{312}, in contrast to anergy mediated by the native peptide, was not accompanied by enhanced responsiveness to exogenous IL-2, suggesting that distinct elements of the anergic phenotype may have different qualitative thresholds for elicitation. From a molecular model of the predicted interactions between the TCR of HA1.7 (31) and the HA306-318/DRB1*0101 complex (17, 18) K^{315} appears to contact glutamic acid at position 94 in the TCR α-chain. Mutation of E94 to either alanine or lysine resulted in the loss of recognition of the wild-type peptide, and stimulation with HA^{315} analogues, including D^{315} and E^{315}, failed to restore recognition. The introduction of these reciprocal mutations at position 315 may result in structural modification at a distant site in the peptide. Similarly, the effects of the amino acid substitution at position 315, which we report here, may also arise by modifying contacts between other positions in the peptide and the TCR.

Since the HA analogues used in this study bind with similar affinity to cell-surface DRB1*0101 molecules (19, 20), either altered intracellular signaling mediated through ligation of the TCR or a lack of costimulatory activity could account for the failure of certain HA analogues to stimulate proliferation in the presence of APCs (32–34). It has been suggested that quantitative differences in the threshold requirements for IL-2 and IL-3 production are influenced by costimulatory signals (32). This is also supported by the observation that the addition of IL-1 was able to restore the loss of proliferation in Th2 cells induced by an altered peptide ligand (15). In contrast, since anergy in our system is generated in the absence of costimulation, it is likely that the qualitative differences in the anergic phenotype induced by the altered TCR ligands occurs as a consequence of changes in the threshold of TCR-mediated signal transduction. Two independent studies have identified a common biochemical mechanism for modified TCR signaling by altered peptide ligands (33, 34). The altered peptide ligands failed to induce ζ chain phosphorylation, which resulted in the inability of ZAP-70 to associate with the TCR complex following transduction of signals from the TCR. Activation of selective intracellular signaling pathways may arise as a consequence of increased peptide concentration and this is indirectly supported by the dose-dependent induction of anergy by H^{315}. In this case increasing the density of the ligand for TCR a "null" signal changes to one capable of inducing partial activation that is not overridden by restimulating with native peptide at higher concentrations or in the presence of different types of APCs. Alternatively, alterations in the affinity or conformation of the ligand, either by the substituted amino acid itself adopting a different orientation or modifying the interaction between TCR at a remote residue, may lead to differential signaling.

Differences in the susceptibility of murine Th2 cells to tolerance induction in terms of Ag-dependent proliferation and responsiveness to IL-4 have been reported (e.g., 8, 10, 13, 14). However, even those Th2 cells that can be rendered anergic retain the capacity to produce IL-4 on restimulation (10). Similarly, anergic murine Th0 cells can still produce IL-4, despite losing the ability to secrete IL-2 (7). This finding may be explained by in vitro experiments in which it was observed that increased protein kinase A activity stimulated IL-4 but inhibited IL-2 expression through suppression of the IL-2 promoter via a binding site that is absent in the IL-4 promoter (35–37). However, for the human Th0 cells studied in this report we failed to observe selective down-regulation of either Th1 or Th2 cytokine production in response to stimulation with any of the peptide analogues in the absence of costimulation. The inability of changes in the threshold of signaling to uncouple IL-4 and IFN-γ production suggests that the regulation of these genes may be under multiple control pathways or have less stringent requirements for costimulation. In support of this is the report that IFN-γ production was only partially inhibited in anergic murine Th1 clones (4).

Our results demonstrate that some peptide analogues containing substitutions that alter the affinity of the interaction between peptide and TCR behaved as incomplete agonists for Th0 cells, failing to stimulate clonal expansion, but capable of delivering signals such that they become anergic to restimulation with the native peptide. Different thresholds of signaling failed to uncouple IL-4 and IFN-γ secretion, nevertheless, in response to stimulation with altered TCR ligands, in the absence of costimulation, dissociation of proliferation from cytokine production was achieved. These findings have implications in peptide-based immunotherapy where it may be desirable to alter qualitative parameters of immune responses, such as those that occur in allergic or autoimmune diseases, by the inhibition or modulation of selected effector functions mediated by specific T cell subsets (e.g., 38–40).

Acknowledgment

The authors thank Dr. Gerard Hoyne for critical review of the manuscript.

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Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 8884–8888, November 1990
Medical Sciences

Induction of specific clonal anergy in human T lymphocytes by *Staphylococcus aureus* enterotoxins

(T-cell tolerance/bacterial toxins/T-cell membrane modulation)

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Communicated by Hugh O. McDevitt, August 17, 1990

ABSTRACT **The exotoxins produced by certain strains of *Staphylococcus aureus* are able to stimulate powerful polyclonal proliferative responses and to induce nonresponsiveness by clonal deletion of T lymphocytes expressing the appropriate T-cell antigen receptor Vβ gene products. This paper examines the ability of *S. aureus* enterotoxins to modulate the responsiveness of human CD4$^+$ T lymphocytes with defined antigen specificity. It was observed that certain *S. aureus* toxins were able to activate and induce anergy in hemagglutinin-reactive T cells expressing Vβ3$^+$ elements. After exposure to *S. aureus* enterotoxins A, B, and D in the absence of antigen-presenting cells, the T cells failed to respond to their natural ligand presented in an immunogenic form, despite enhanced proliferation to exogenous interleukin 2. The *S. aureus* toxin-induced anergy was associated with modulation of T-cell membrane receptors; down-regulation of the T-cell antigen receptor was concomitant with enhanced expression of CD2 and CD25. Interestingly, CD28 was increased only on stimulation, suggesting this protein may be differentially expressed by activated and anergic T cells. These results indicate that bacterial toxins are able to induce antigen-specific nonresponsiveness in human T cells, the application of which may be relevant in the regulation of T cells expressing a particular family of Vβ gene products.**

The staphylococcal enterotoxins (1, 2) and certain endogenously derived proteins such as Mls (3, 4) are members of a family of antigens termed "superantigens," based on their ability to stimulate powerful polyclonal proliferative responses of murine and human T lymphocytes bearing particular T-cell antigen receptor (TCR) Vβ gene products (4–7). Additionally, superantigens are also able to induce nonresponsiveness in murine T cells either by clonal deletion (5) or functional inactivation (8). With the development of *in vitro* experimental systems, it has been possible to demonstrate that occupancy of the TCR by peptidic fragments of antigen complexed with class II major histocompatibility complex (MHC) molecules, in the absence of additional signals (costimulatory activity), is able to induce antigen-specific anergy (9–12). However, direct evidence to support clonal anergy as an operational mechanism in the development and maintenance of tolerance to either self or extrinsic antigens *in vivo* has been difficult to obtain. The results of recent experiments examining T-cell tolerance to nonlymphoid-expressed MHC molecules (13, 14) or to the self superantigen Mls-1^a (8) suggest that nonresponsiveness, in certain instances, may be accounted for by functional inactivation. Similarity between the functional characteristics of these *in vivo* experimental models and those of peptide-specific T-cell anergy induced *in vitro* (9–12) prompted us to investigate the ability of *Staphylococcus aureus* enterotoxins to induce antigen-specific nonresponsiveness in cloned human CD4$^+$ T cells specific for the carboxyl terminus of influenza virus hemagglutinin (HA), residues 307–319 [HA-(307–319)] (15, 16). In this report we demonstrate that the *S. aureus* toxins which were able to stimulate proliferation could also render the HA-reactive T cells nonresponsive to an immunogenic challenge of viral antigen and that the mechanism of nonresponsiveness is associated with modulation of T-cell membrane proteins.

MATERIALS AND METHODS

Antigens. Staphylococcal enterotoxins A, B, C1, C2, C3, and D (SEA, SEB, SEC1, SEC2, SEC3, and SED) were purchased from Toxin Technology (Madison, WI) or Serva Fine Biochemicals (New York). The HA peptide (residues 307–319) was synthesized using standard solid-phase methods on an Applied Biosystems model 430A synthesizer, purified by reversed-phase HPLC, and analyzed by amino acid analysis as described (16). This peptide was generously provided by J. Rothbard (ImmuLogic).

Antibodies. For flow cytometric analysis, fluorescein-conjugated murine monoclonal antibodies, anti-Leu5b (CD2), anti-Leu4 (CD3), anti-Leu3a (CD4), anti-interleukin 2 (IL-2) receptor (CD25), and fluorescein isothiocyanate-conjugated mouse IgG1 control were purchased from Becton Dickinson. The murine monoclonal antibodies anti-CD28 (9.3; ref. 17) and anti-CD3 were generously provided by J. Ledbetter (Oncogen, Seattle, WA) and H. Spits (DNAX), respectively.

Cloned Human Antigen-Reactive T Lymphocytes. The isolation and characterization of the cloned human T cells reactive with HA-(307–319) have been reported in detail elsewhere (15). Briefly, T cells activated with immunochemically purified HA were resuspended in RPMI 1640 medium (GIBCO) supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), 2 mM L-glutamine, and 5% (vol/vol) screened human AB$^-$ serum and cloned by limiting dilution in the presence of autologous irradiated peripheral blood mononuclear leukocytes, IL-2 [10% (vol/vol) Lymphocult T; Biotest Folex, Frankfurt, F.R.G.], and antigen. Growing T cells were expanded by cyclic stimulation with antigen and filler cells every 7 days and with IL-2 every 3 or 4 days. Prior to their use in experiments the T cells were allowed to rest for 7 days after the last exposure to antigen and filler cells.

Induction of T-Cell Nonresponsiveness. T cells (10^6 cells per ml) were incubated for 16 hr with the *S. aureus* toxins (0.5 μg/ml) or HA-(307–319) (50 μg/ml; ref. 9). Control cultures

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Abbreviations: APC, antigen-presenting cell; HA, influenza virus hemagglutinin; MHC, major histocompatibility complex; TCR, T-cell antigen receptor; IL-2, interleukin 2; SE, staphylococcal enterotoxin.

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of T cells in medium or of T cells activated with insolubilized anti-CD3 antibody (12 μg/ml) and IL-2 were performed in parallel. The cells were washed extensively after the pretreatment before determining their ability to respond to either an immunogenic challenge of antigen [HA-(307–319)] and antigen-presenting cells (APCs) or IL-2 (10%).

Proliferation Assays. Cloned T cells (10^5 cells per ml) were stimulated with HA-(307–319) (1.0 μg/ml) or the *S. aureus* toxins at the concentrations as indicated in the figures, in the presence of mitomycin C-treated murine fibroblasts expressing HLA-DR1 (10^5 cells per ml; ref. 16) as a source of APCs, or in IL-2 alone. After 60 hr of incubation, [*methyl*-^{3}H]thymidine (1 μCi/ml; 1 Ci = 37 GBq; Amersham) was added and the cultures were harvested onto glass fiber filters 8–16 hr later. Proliferation as correlated with [^{3}H]thymidine incorporation was measured by liquid scintillation spectroscopy. The results are expressed as mean cpm for triplicate cultures. In all cases the standard error of the mean was <20%.

Fluorescence Flow Cytometry. T cells were stained directly with saturating concentrations of fluorescein-conjugated murine monoclonal antibodies, anti-Leu5b (CD2), anti-Leu4 (CD3), anti-Leu3a (CD4), or anti-IL-2 receptor (CD25) using a mouse IgG1 fluorescein isothiocyanate-conjugated control, or indirectly with 9.3 (CD28). Viable cells, identified by their ability to exclude propidium iodide, were analyzed by flow cytometry using a FACScan (Becton Dickinson). The cell population was analyzed by gating on the volume and light-scatter characteristics.

RESULTS

The Proliferative Response of Cloned HA-Reactive T Cells (HA1.7) to the *S. aureus* Enterotoxins. Distinct patterns of responsiveness were observed when T cells of clone HA1.7 were cultured alone or with APCs or IL-2 in the presence of the *S. aureus* enterotoxins, over a broad concentration range (Fig. 1). These cloned cells express αβ TCRs bearing Vβ3 gene products (M. J. Owen, personal communication). SEA at 0.5 μg/ml in the presence of APCs induced a weak but reproducible proliferative response (Fig. 1*a*). Although mediated at different concentrations, with SED (Fig. 1*f*) being two orders of magnitude more potent, the effects of this toxin and SEB (Fig. 1*b*) on the T cells were similar. Interestingly, proliferation in response to the natural ligand HA-(307–319), in association with DR1, was always at least 5-fold greater than that induced by any of the *S. aureus* toxins tested. At the appropriate concentration, SEB or SED alone induced T-cell proliferation in the absence of APCs; nevertheless, the response was decreased compared to that observed when APCs were present. In parallel the doses of toxin capable of inducing proliferation decreased responsiveness to exogenous IL-2. The patterns of response to SEC1, -2, and -3 were generally similar in that these toxins failed to induce T-cell proliferation even in the presence of APCs (Fig. 1 *c–e*).

Induction of HA-Specific Nonresponsiveness After Exposure to *S. aureus* Toxins. Preincubation with SEA, SEB, and SED for 16 hr in the absence of APCs induced nonresponsiveness in the T cells such that they were unable to proliferate in response to an immunogenic challenge of HA presented by murine fibroblasts expressing HLA-DR1 (Fig. 2). When peripheral blood mononuclear leukocytes or Epstein–Barr virus-transformed B cells were used as a source of APCs, the enterotoxin-pretreated T cells also failed to respond to specific antigen. This suggests that the nonresponsiveness observed in the presence of the DR1$^+$ transfectants is not the result of a lack of accessory-cell costimulatory activity. In the presence of APCs, the toxins are also able to reduce the

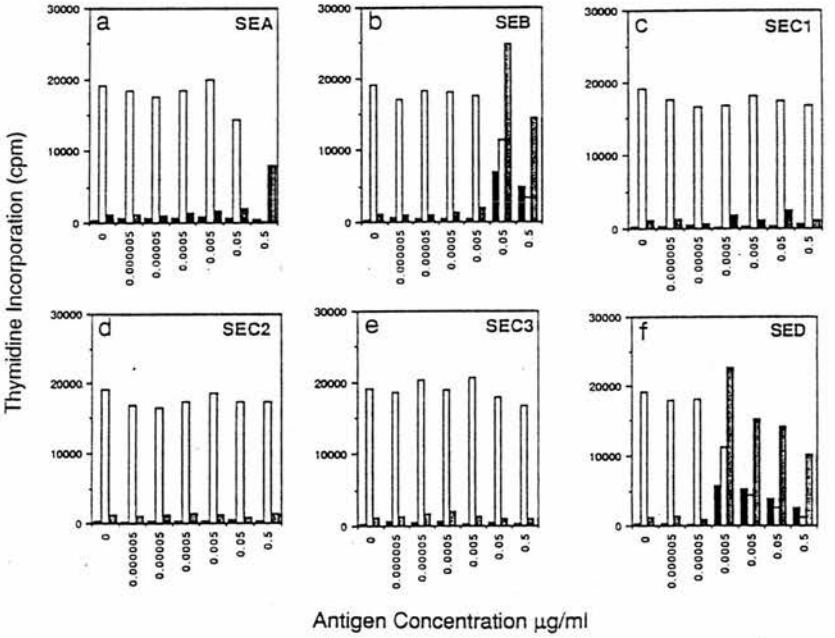

FIG. 1. Effect of *S. aureus* toxins on the proliferative response of HA1.7. Cloned T cells were stimulated with increasing concentrations of staphylococcal enterotoxins, SEA (*a*), SEB (*b*), SEC1 (*c*), SEC2 (*d*), SEC3 (*e*), and SED (*f*) alone (solid bars), with IL-2 (open bars), or with mitomycin C-treated murine fibroblasts expressing HLA-DR1 (stippled bars). The control response of the T cells to HA-(307–319) at an optimum concentration of 1 μg/ml was 96,070 cpm (± 5%) (mean ± SEM).

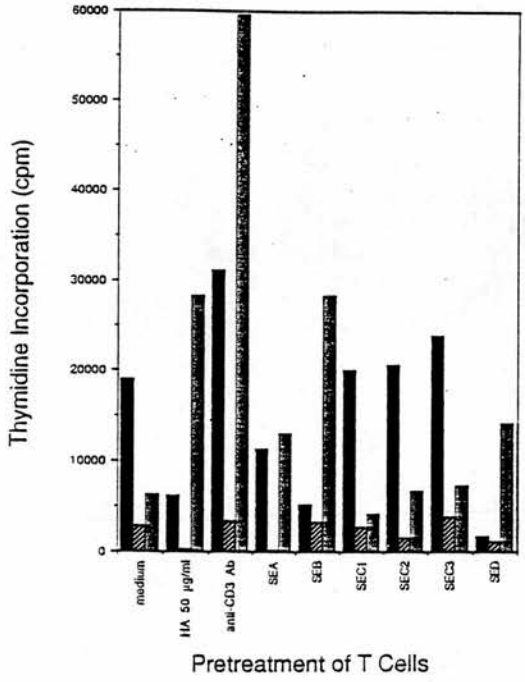

FIG. 2. Functional inactivation of T-cell clone HA1.7 after exposure to *S. aureus* toxins. T cells were exposed to the *S. aureus* toxins under conditions that induce unresponsiveness (as indicated). In the control cultures, T cells were incubated in medium alone or with the HA peptide or anti-CD3 antibody and IL-2. From each group of treatments T cells were assayed for their ability to respond to an immunogenic challenge of HA-(307–319) and accessory cells (mitomycin C-treated murine fibroblasts expressing HLA-DR1; solid bars), accessory cells alone (hatched bars), or IL-2 (stippled bars).

response of the T cells to specific antigen, although higher concentrations are required. A similar state of specific anergy resulted when the T cells were exposed to a supraimmunogenic concentration of free HA peptide but not to a peptide of unrelated sequence (e.g., see Fig. 4*c*). Unlike the activated T cells, both HA-peptide- and toxin-tolerized cells were refractory to an immunogenic challenge for up to 5 days. Concomitant with the loss of antigen-specific nonresponsiveness, a reciprocal enhancement of the proliferative response to IL-2 was demonstrated. As observed in activation, the tolerogenic effects of the toxins could be ranked as SED > SEB > SEA. In contrast, neither antigen- nor IL-2-dependent proliferation was modulated by exposure of the T cells to SEC1, -2, or -3 (Fig. 2).

Phenotypic Modulation Accompanying *S. aureus* Toxin and HA-Peptide-Induced Nonresponsiveness. To determine whether or not nonresponsiveness was due to receptor modulation, the T cells were analyzed by flow cytometry. Changes in phenotype observed after exposure to SEA, SEB, and SED were comparable (Fig. 3). The reduced expression of CD3 (Fig. 3*a*) was accompanied by up-regulation of CD2 (Fig. 3*b*) and CD25 (Fig. 3*c*). The TCR was modulated in parallel with CD3, as determined by staining with the monoclonal antibody WT31, which recognizes the $\alpha\beta$ TCR (data not shown). Activation with insolubilized anti-CD3 antibody and IL-2- or HA-peptide-induced anergy revealed similar changes in the phenotype. Membrane levels of CD4 were unaltered by exposure to HA-(307–319) or most of the *S. aureus* toxins tested, the exception being SEA, which enhanced CD4 expression, although the effect was marginal (Fig. 3*d*). The level of CD28 was marginally, but reproducibly, down-regulated (20–35%, $n = 6$) in toxin- and HA-peptide-induced anergy, whereas activation with anti-CD3 antibody and IL-2 markedly enhanced the expression (Fig. 3*e*). The phenotype of the T cells after pretreatment with SEC1, -2, and -3 were indistinguishable from the medium control.

To determine whether or not functional inactivation paralleled phenotypic modulation, the T cells were exposed to increasing concentrations of SEB and the loss of antigen-dependent proliferation was compared to the expression of CD3 and CD25. At concentrations of SEB >0.05 μg/ml, the down-regulation of CD3 (Fig. 4*a*) correlated with functional inactivation (Fig. 4*c*). Similarly, no changes in the expression of CD25 (Fig. 4*b*) were observed in the presence of SEB at concentrations that failed to induce anergy. Control cultures of T cells tolerized with HA-(307–319) revealed the same phenotypic modulation, whereas an irrelevant peptide derived from the group II allergen of dust mite, residues 36–60, had no effect.

DISCUSSION

The present study demonstrates that human T cells of defined antigen specificity exposed to certain *S. aureus* toxins, in the absence of accessory cells, become anergic to an immunogenic challenge by their natural ligand but retain responsiveness to IL-2. Distinct patterns of proliferation were observed when cloned human Vβ3$^+$ T cells specific for HA were cultured with the *S. aureus* toxins (SEA to -D) under stimulatory conditions. SEA, SEB, and SED induced proliferation in the presence of APCs, albeit with different potencies. Murine and human T cells expressing Vβ3 elements are able to interact specifically with SEB (6, 7); therefore, it was not surprising that this toxin is able to stimulate the HA-specific T cells. Human CD4$^+$ and CD8$^+$ T-cell clones activated by SEA and SEB have been identified (18), and since these toxins have ≈30% sequence identity (19, 20), a common sequence may be present that allows binding to the Vβ gene products expressed by the two subsets of T cells. An immunologically related functional site has tentatively been localized at the amino terminus of SEA within residues 1–27 (ref. 21). However, the comparable region in SEB shows only limited homology and, therefore, it is unlikely that this sequence contains the active site that triggers the T cells used in this study. Although, of the *S. aureus* toxins, SEC1 and SEB (22) are the most homologous, the Vβ3$^+$ T cells would appear to bind only the latter. Weak stimulation of the T cells by SEB and SED in the absence of APCs was observed. Although bacterial toxins appear to require no cellular processing to stimulate T cells (1, 18) and activated human T cells express class II MHC molecules, the inability of the T cells to provide adequate accessory signals may account for the suboptimal activation.

Interestingly, in the absence of APCs, those *S. aureus* toxins capable of inducing proliferation were able to modulate antigen recognition by the cloned T cells; such that the T cells failed to respond to an immunogenic challenge of the appropriate ligand. The failure to respond to antigen was not due to cytolysis since IL-2 responsiveness was enhanced. This phenomenon of T-cell nonresponsiveness is similar to that induced by free antigen in peptidic form (9) or antigen presented by chemically modified accessory cells (10–12). This finding demonstrates that extrinsic superantigens are able to functionally inactivate the response of human T cells to their natural ligand, in this case HA. Our observations parallel those reported for specific tolerance to Mls-1^a, the self superantigen in adult Mls-1^b mice (8). The observation that Vβ6$^+$ peripheral T cells are excluded after stimulation

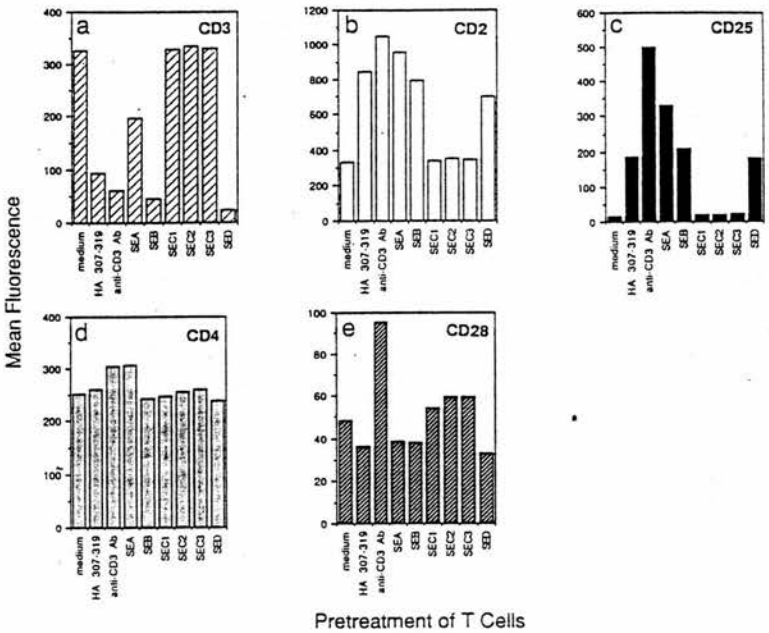

FIG. 3. Phenotypic modulation of the T cells functionally inactivated by *S. aureus* toxins. T cells were exposed to the *S. aureus* toxins under conditions that induce unresponsiveness. Membrane expression of CD3 (*a*), CD2 (*b*), CD25 (*c*), CD4 (*d*), and CD28 (*e*) was examined by flow cytometry and compared to control cultures of T cells in medium alone, tolerized with HA-(307–319), or activated with anti-CD3 antibody and IL-2.

with SED is compatible with the ability of enterotoxins to induce nonresponsiveness of human T cells (6). In experimental animal models, tolerance to SEB may also result from the physical elimination of T cells (5). The direct interaction of SEB with the TCR (4, 18) or through MHC class II molecules on APCs could account for the clonal deletion. From serological inhibition studies, it appears that, in contrast to the HA peptide (23), under conditions that induce anergy, the toxins bind to TCR independently of MHC class II molecules and transduce negative signals directly to the T cells (unpublished observations). However, whether or not bacterial superantigens bind directly to TCR remains controversial (1, 4, 18).

The induction of anergy by the *S. aureus* toxins resulted in changes in the phenotype of the T cells. The Ti–CD3 antigen receptor complex was modulated from the cell surface after exposure to SEA, SEB, or SED and correlated with the failure of the T cells to proliferate in response to specific peptides. However, after overnight activation with anti-CD3 and IL-2, unlike the HA peptide (24) and despite the down-regulation of membrane Ti–CD3, an immunogenic challenge still elicited proliferation. The rapid recovery of Ti–CD3 after activation may account for the antigen-dependent response. The longevity of anergy (9, 10) and the lack of Ti–CD3 on the cells tolerized by chemically modified APCs and antigen (12) indicate that anergy is associated with complex molecular regulation and is not solely the result of TCR modulation. In the nonresponsive T cells, the expression of TCR and CD25 is reciprocal. The up-regulation of CD25 and the subsequent increased IL-2 responsiveness of the anergic T cells may lower the effective IL-2 concentration and account for the apparent suppressor activity of SEA (25) without invoking the generation of an additional regulatory cell type.

There was no comodulation of CD4 with CD3 from the T-cell membrane in the anergic T cells, which suggests that for these cloned T cells CD4 is not structurally part of the antigen-recognition complex (26). However, the interactions between Ti–CD3 and CD2 appear to be considerably more complex. Coprecipitation studies have demonstrated that ≈40% of membrane CD2 is physically associated with Ti–CD3 (27). Interestingly, in the experiments reported here the expression of CD2 and Ti–CD3 are reciprocal in both toxin-mediated anergy and activation. Their relationship is further complicated by the observation that cholera toxin modulates only Ti–CD3 on the human T-cell lymphoma Jurkat (28). Collectively, these findings suggest that two populations of CD2 may exist that are modulated independently and may have different functional roles in the regulation of T-cell activation. The regulation of CD28 expression was intriguing by virtue of its association with an alternative pathway of activation independent of antigen recognition by the Ti–CD3 complex (29). Although, marginally down-regulated in anergy, enhancement occurred in activation. It has been postulated that CD28 may be the receptor for costimulatory activity that determines the outcome of tolerance or activation after occupancy of the TCR, based on the molecular analysis of peptide-induced anergy (12). Our experiments were not designed to address this issue; nevertheless they demonstrate that CD28 is differentially expressed in activated (anti-CD3) and anergic [HA-(307–319), SEA, SEB, and SED] T cells (Fig. 3).

S. aureus toxins react with particular Vβ gene products of TCRs (4–7) and, as is reported here, are also able to inactivate T cells such that they fail to respond to their natural ligand. It has been observed that carboxymethylation, although not altering antigenicity, which remains equal to that of the native molecule, removes the enterotoxic properties of

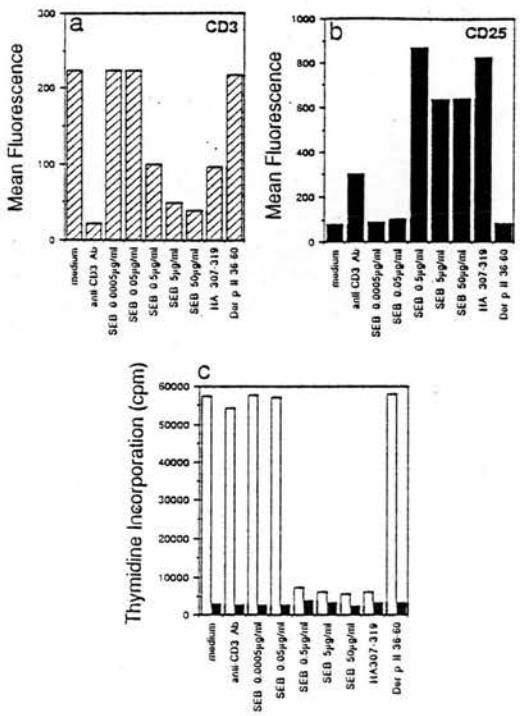

FIG. 4. Dose dependence of functional inactivation and phenotypic modulation. After exposure to SEB at increasing concentrations, T-cell membrane expression of CD3 (*a*) and CD25 (*b*) were compared to their responsiveness to an immunogenic challenge (*c*) of HA-(307–319) and DR1$^+$ APCs (open bars) or DR1$^+$ APCs alone (solid bars). Control cultures of T cells in medium alone, activated with anti-CD3 or pretreated with tolerogenic concentrations of HA-(307–319) or an irrelevant peptide derived from dust mite (Der pII 36–60) were examined.

SEA (32). The ability of the enterotoxins to induce nonresponsiveness of T cells in the presence of APCs, although less efficiently than in their absence, suggests they will retain this property *in vivo* (8). This raises the possibility of using superantigens as tolerogens to inactivate subpopulations of T cells that express TCR with common features. This approach would be of particular relevance in certain autoimmune diseases where the diversity of TCR is limited (30, 31). Furthermore, by genetic manipulation both the tolerogenic activity and affinity of enterotoxins for TCRs may be enhanced and, therefore, have potential as an alternative method of therapeutic intervention.

We are grateful to Dr. Ronald Schwartz for his suggestion to investigate the modulation of CD28 in T-cell anergy. Helpful scientific discussions with Drs. G. Fathman, M. Gefter, and H. McDevitt are acknowledged. R.E.O'H. is the recipient of a Wellcome Senior Fellowship in the Clinical Sciences. This research was supported in part by the Medical Research Council, London.

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Reprinted from Nature, Vol. 308, No. 5954, pp. 72-74, 1 March 1984

Essential requirement for major histocompatibility complex recognition in T-cell tolerance induction

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The induction of T-cell responses involves the recognition of extrinsic antigen in association with antigens of the major histocompatibility complex (MHC), in mice and man, with different T cells recognizing antigen in association with either class I (H-2K/D, HLA-A, B, C) or class II (Ia, HLA-D/DR) MHC antigens[1-5]. However, the requirement of MHC recognition in the induction of immunological tolerance remains ill defined. With human T helper clones recognizing synthetic peptides of influenza haemagglutinin (HA-1), we have investigated the nature of antigen-induced stimulation[6], and antigen-induced antigen-specific unresponsiveness, immunological tolerance[7]. Tolerance is not due to cell death, as the cells remain responsive to interleukin-2 and is associated with the loss of T3 antigen from the cell surface[8]. Using monoclonal antibodies to the non-polymorphic regions of human class II antigens to inhibit the induction of T-cell tolerance we report here that induction of tolerance requires the recognition of MHC antigens.

Since T lymphocyte clones can be maintained in long-term culture it is now possible to analyse their mechanisms of antigen recognition and regulation in more detail than previously. We have produced a number of human influenza-specific T-cell clones with a diversity of function, such as help[9], suppression[10] and lymphokine production[11]. They have been analysed for the fine specificity of their response to antigen. Clones HA1.4 and HA1.7 used in this study respond to a 24 amino acid peptide derived from the C-terminal region of HA-1 (residues 306–329) termed peptide 20, or p20† (ref. 12). This is an immunodominant region, as many clones raised with the intact haemagglutinin molecule react to this region[13].

Depending on the nature and concentration of specific antigen, duration of culture and the presence of antigen-presenting cells, these clones can be either induced to proliferate, or can be rendered unresponsive[7]. Hence these clones are suitable for investigating the nature of the 'signals' necessary for T-cell activation or tolerance induction. Since recognition of MHC is of paramount importance for activation of T cells, we wanted to determine whether or not this was also the case for the induction of tolerance.

The human *HLA-D* region, corresponding to the mouse *H-2 I* region, controls a number of different products, which have been described serologically under a variety of names. There are three *HLA-D* groups (reviewed in refs 2, 14, 15). *HLA-DR* and *HLA-SB* correspond to the mouse *I-E* region, as judged by sequence homology and serological cross-reactivity. *HLA-DC/DS* (also known as *MT* and *MB, LB*), corresponds to the murine *I-A* subregion. There is evidence that HLA-DR (ref. 16), SB (ref. 17) and DC (D. D. Eckels, personal communication) all act as MHC targets of T-cell recognition by various clones.

The requirement for class II MHC determinants in T-cell activation can be demonstrated either by the addition of antigen-presenting cells bearing the appropriate MHC specificities or by the inhibition of T-cell proliferation by anti-class II antibodies specific for the various subregions[18]. The former approach cannot be used as tolerance is more readily induced in the absence of antigen-presenting cells[7], and so serological inhibition, using rabbit anti-human Ia, and mouse monoclonal antibodies directed against class II antigens, either HLA-DC/DS or HLA-DR, was used. As controls, monoclonal anti-HLA-A, B, C, anti-β_2-microglobulin, anti-T4 and anti-T3 antibodies were used.

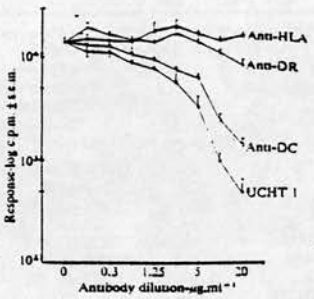

Fig. 1 Inhibition of antigen-induced proliferative response of cloned helper T cells reactive with HA peptide 20 by anti-HLA class II antibodies. Cells of HA1.7 (2.5×10^4 per ml) were stimulated with p20 (1.0 μg ml^{-1}) in the presence of irradiated E$^-$ cells (2.5×10^4 per ml) in 96-well round-bottom culture plates. Antibodies (2A1, □; DA-2, ▲; UCHT1, ■; and SG465, ○) were added at the start of the cultures in the concentrations as indicated (0.15–20 μg ml^{-1} in doubling dilution) and left in for the duration of the culture. Proliferation was assayed as described in the legend to Table 1. The background responses of cloned T cells in the absence of irradiated E$^-$ cells was <150 c.p.m. as was that of E$^-$ cells alone cultured with antigen.

The proliferative response of clone HA1.7 is inhibited by anti-class II, especially anti-DC/DS and thus involves recognition of HLA-DC/DS (Fig. 1). Anti-HLA-A, B, C had no effect, anti-HLA-DR had a minimal effect. This is presumably because there is a degree of cross-reactivity between the non-polymorphic components of HLA-DR and HLA-DC, as has been previously demonstrated biochemically[19,20].

One possible interpretation of the inhibition mediated by anti-HLA-DC was that it was due to a direct effect on T cells in the absence of antigen. This was excluded by experiments in which T cells were pretreated with antibodies and then washed before an antigenic challenge (Table 1). In these conditions, only anti-T3 inhibited T-cell proliferation. No enhancement or inhibition of proliferation was detected with either monoclonal or rabbit anti-class II antibodies. Therefore it would seem that the anti-class II antibodies mediated their effects by inhibiting antigen-induced activation[18], and not by direct effect on the T cells.

Since human T-cell clones express class II antigens[11,21,22] it was possible to investigate whether anti-class II antibodies could inhibit the induction of tolerance. Pretreatment of the clones with anti-HLA-DC/DS and rabbit anti-Ia blocked tolerance induction completely, whereas anti-HLA-DR had only a marginal effect (Table 2). Anti-MHC class I had no effect. These results indicate that tolerance involves recognition of MHC class II antigens. To determine more precisely which MHC class II antigens were involved, dose–response curves of anti-HLA-DC/DS and anti-HLA-DR antibodies on tolerance induction are compared. This was necessary as immunoprecipitation analysis has shown that the anti-DR monoclonal DA2 cross reacts weakly with DC/DS molecules[19,20], which would explain its partial effects on the induction of proliferation and tolerance. However, at lower concentrations (1.25 μg ml^{-1}), only the anti-

Table 1 Effect of antibodies on the induction of unresponsiveness in the absence of specific antigen

Pretreatment		Response of cloned T helper cells, (c.p.m. ± s.e.m.)	
Antibody	Antigen	E^- + peptide 20	TCGF
—	—	11,454 ± 856	3,751 ± 497
—	p20	506 ± 104	5,677 ± 566
—	p11	10,924 ± 1,103	3,356 ± 331
Anti-HLA (2A1)	—	8,881 ± 804	4,309 ± 499
Anti-DC (SG465)	—	12,453 ± 1,862	2,264 ± 226
Anti-DR (DA2)	—	8,790 ± 329	2,744 ± 340
Raia	—	8,478 ± 1,123	2,994 ± 445
Anti-T3 (UCHT1)	—	698 ± 138	5,279 ± 267
Anti-β_2M (M8)	—	11,926 ± 634	4,018 ± 482
Anti-T4 (MT321)	—	7,408 ± 612	3,406 ± 675

T lymphocyte clones reactive with the synthetic peptides of influenza virus haemagglutinin (HA) were isolated as described previously[9]. Briefly, peripheral blood mononuclear leukocytes (PBL) were stimulated for 6 days with HA (0.1 $\mu g\,ml^{-1}$). Following enrichment on a discontinuous Percoll gradient (Pharmacia) the lymphoblasts were resuspended in RPMI-1640 (Gibco) supplemented with 10% pooled A^- serum and 20% T-cell growth factor (TCGF) and cloned by limiting dilution. Lymphoblasts were plated one cell every third well in sterile 60-well Microtest II trays (Falcon) with 10^4 irradiated (2,500 rad) autologous (PBL) and 0.1 $\mu g\,ml^{-1}$ of HA. At 7 days, growing clones were transferred to 96-well microtitre trays and subsequently to 24-well plates. At each transfer the clones received fresh TCGF every 3–4 days and irradiated histocompatible PBL and influenza virus (A/Texas/1/77) every 7 days. TCGF was prepared from tonsillar lymphocytes ($1 \times 10^6\,ml^{-1}$) stimulated with 0.1% purified phytohaemagglutinin (Difco) in complete culture medium supplemented with 2.5% pooled A^- serum. Before use in experiments the clones were rested for 7 days after the addition of filler cells. To induce tolerance, cloned T cells were cultured at 10^5 per ml in round bottom 96-well plates for 16 h with HA peptides (50 $\mu g\,ml^{-1}$) or monoclonal antibodies (5 $\mu g\,ml^{-1}$) for 16 h. Intact virus or haemagglutinin at high concentrations are toxic in culture and do not induce tolerance. Rabbit anti-Ia was used at a 1:50 dilution. After washing the cloned T cells (2.5×10^4 per ml) were cultured with irradiated histocompatible sheep erythrocyte rosette negative (E^-) lymphocytes (2.5×10^4 per ml) that had been pulsed overnight with 1.0 $\mu g\,ml^{-1}$ of p20. Following 60 h incubation the cultures were pulsed for 8–16 h with 1.0 μCi of ^{3}H-thymidine (^{3}HTdR) (Amersham) and collected onto glass fibre filters. Proliferation as correlated with ^{3}H-TdR incorporation was measured by liquid scintillation spectroscopy. The results are expressed as mean counts per min (c.p.m.) ± s.e. of the mean of triplicate cultures. UCHT1 is specific for the T3 antigen present on all mature functional T cells[31]. 2A1 is against a nonpolymorphic determinant of HLA class I[32]. M8 is against β_2-microglobulin[33]. MT321 is against the helper/inducer subset (P. Rieber, personal communication), and rabbit anti-Ia is broadly reactive with human Ia-like antigens[34]. DA-2 is reactive with a monomorphic determinant of HLA-DR (ref. 23) and SG465 is an antibody reactive with HLA-DC determinants[27].

HLA-DC/DS monoclonal inhibits tolerance induction (Fig. 2), and thus the type of class II molecule recognized is the same for both immunity and tolerance.

Analogous results have been obtained with two anti-HLA-DR reagents, DA2 (ref. 23), CA2.06 (ref. 24), and three anti-HLA-DC reagents, SDR 4.1 (ref. 25), HIG 78 (ref. 26) and SG 465 (ref. 27). To confirm that MHC involvement in tolerance induction was not unique to clone HA1.7, a second p20 specific clone HA1.4 was also investigated, and showed similar inhibition by the same anti-class II reagents. Questions of cross-reactivity between monomorphic anti-DR or anti-DC antibodies do not affect the major point of this communication, that recognition of MHC class II antigens is of importance in tolerance induction.

However, the experiments reported here do not resolve whether the class II molecules involved in tolerance induction are present on the same T cell, or adjacent T cells. Our previous results have indicated that cloned T cells can present antigen to other T cells, using an 'anti-idiotype' (anti-receptor) clone[10].

Table 2 Anti-Ia antibody inhibits tolerance induction

Pretreatment		Response of cloned T helper cells (c.p.m. ± s.e.m.)	
Antibody	Antigen	E^- + Peptide 20	TCGF
—	p20	674 ± 90	5,730 ± 437
—	p11	9,210 ± 524	4,461 ± 428
Anti-HLA (2A1)	p20	456 ± 82	4,150 ± 773
Anti-DC (SG 465)	p20	8,542 ± 267	2,368 ± 409
Anti-DR (DA2)	p20	2,225 ± 226	2,712 ± 276
Raia	p20	9,700 ± 972	2,697 ± 160
Anti-T3 (UCHT1)	p20	728 ± 137	5,141 ± 1016
Anti-β_2M (M8)	p20	964 ± 258	3,589 ± 773
Anti-T4 (MT 321)	p20	880 ± 123	4,547 ± 71
—	—	10,591 ± 785	3,966 ± 486

Cloned T cells were preincubated with either peptide or antibody prior to assay or antigen-pulsed E^- cells as described in Table 1 legend. Antibody was added 6 h prior to the addition of antigen, and left in for the 16 h of antigen exposure before washing the cells three times. Proliferation was determined as described in the legend to Table 1.

Studies *in vivo* have attempted to examine whether tolerance to minor transplantation antigens is MHC restricted. Matzinger and Waterfield[28] used F_1–parent bone marrow chimaeras, but due to the possibility of antigen reprocessing by F_1 macrophages could not make a definitive assessment. More recently Groves and Singer[29] have used parent-F_1 irradiation chimaeras. Their results suggest that tolerance of cytotoxic T-cell precursors involves MHC recognition, in keeping with the *in vitro* clonal analysis reported here.

The observation that T-cell tolerance involves MHC recognition is compatible with either a single or dual T-cell receptor concept. However, it is not consistent with the concept that T-cell immunization involves recognition of antigen in association with MHC, whereas tolerance involves recognition of antigen alone[30]. If 'positive' induction of a helper clone, to proliferate and help, and 'negative' induction to be tolerant both depend on recognition of the same antigen and MHC product, it is pertinent to ask what factors discriminate between these opposite tendencies. At present antigen concentration and antigen-presenting cells have been shown to be relevant. However, the role of other signals, such as interleukin-1, remain to be elucidated.

We thank J. Knight and S. Smith for technical assistance, Drs P. C. L. Beverley, J. Bodmer, M. Crumpton, S. Goyert, J. Silver, V. van Heyningen for the gift of monoclonal antibodies, Drs N.

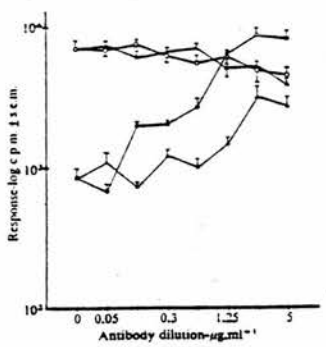

Fig. 2 Dose–response curve of the inhibition of tolerance induction by anti-HLA-DC and anti-HLA-DR antibodies. Cells of HA1.7 were pretreated with various concentrations of antibody (anti-HLA-DC, SG 465[27] ●, ○; anti-HLA-DR, DA2[23] ▲, △) and the antigen specific (●, ▲) and TCGF (○, △) responses assayed as described in the legend to Table 1.

Green and R. A. Lerner for the gift of synthetic polypeptides of influenza haemagglutinin, Dr R. G. Webster for the gift of haemagglutinin, Dr T. Davies and his colleagues at the National Blood Transfusion Centre, Edgware, for assistance and the Imperial Cancer Research Fund and NIH for financial support.

Received 5 August; accepted 12 December 1983.

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Reprinted from THE JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY, St. Louis
Vol. 90, No. 5, pp. 749-756, November, 1992 (Printed in the U.S.A.)

Peptide-induced nonresponsiveness of HLA-DP restricted human T cells reactive with *Dermatophagoides* spp. (house dust mite)

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The activation of CD4+ T lymphocytes, which play a central role in allergic inflammation, depends on the recognition of allergen-derived peptides in association with major histocompatibility complex class II gene products. In this report we demonstrate, at a clonal level, that a component of the T-cell repertoire reactive with Dermatophagoides *spp. (house dust mite) in atopic individuals, is restricted by HLA-DP class II molecules. This supports the recent results emerging from genetic epidemiologic studies that indicate positive associations between the HLA-DP phenotype and immune responsiveness to a variety of common allergens. Our findings also reveal that the T cells restricted by HLA-DP recognize a species-specific epitope located in the group I allergen of* Dermatophagoides pteronyssinus *(residues 101-119). Furthermore, we report that the pretreatment of the T cells restricted by HLA-DP with the* Der p *I peptide renders them nonresponsive to an immunogenic challenge with house dust mite allergen, and the loss of antigen-dependent proliferation is associated with downregulation of membrane expression of the T-cell antigen receptor. The ability to functionally inactivate T cells restricted by HLA-DP, as well as those that recognize allergen in association with HLA-DR class II molecules, suggests that desensitization with allergen-derived peptides may have therapeutic potential in the management of allergic diseases irrespective of their HLA class II association. (J ALLERGY CLIN IMMUNOL 1992;90:749-56.)*

Key words: Peptide, T cells, MHC, HLA-DP, Dermatophagoides *spp., house dust mite,* Der p *I, desensitization*

It is now well documented that CD4+ T lymphocytes are critical for the induction of both the specific (IgE) and nonspecific (polymorphonuclear granulocytes) effector mechanisms of allergic inflammation.[1-3] The activation of CD4+ T cells requires the formation of molecular interactions between T-cell receptor, peptide fragments of antigen, and major histocompatibility complex (MHC) class II gene products.[4-6] Therefore the importance of MHC molecules in the regulation of immune responses prompted genetic epidemiologic studies in an attempt to establish an association between human leucocyte antigen (HLA) phenotype and allergic immune responses to

Abbreviations used

MHC:	Major histocompatibility complex
IL-2:	Interleukin-2
HDM:	House dust mite
PBMC:	Peripheral blood mononuclear cell
[^{3}H]TdR:	Tritiated methyl thymidine
EBV:	Epstein-Barr virus
cpm:	Counts per minute
Der p I:	Group I allergen of *Dermatophagoides pteronyssinus*
APC:	Antigen presenting cell

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Supported by grants from the Wellcome Trust, England, the Medical Research Council, England, and the Imperial Cancer Research Fund, England.
Received for publication March 17, 1992.
Revised June 8, 1992.
Accepted for publication June 9, 1992.
[c]R. E. O'Hehir is the recipient of a Wellcome Senior Research Fellowship in Clinical Sciences.
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environmental allergens. The initial analyses concentrated on pollen antigens, and consequently it was demonstrated that the production of IgE after exposure to the ragweed pollen antigens *Amb a* V, *Amb t* V, *Amb p* V, and *Amb a* VI occurred in individuals expressing DRB1*1501 and DRB5*0101 (DR2 Dw2, DR51*) gene products.[7-10] With the combined approaches of sequence-specific oligonucleotide typing and antigen-specific T-cell clones,[10-12] these HLA associations for ragweed pollen have been confirmed. The extension of these studies on the linkage between HLA and atopic disease established that specific immune responses to the rye grass antigens of *Lolium perenne (Lol p)* I, II, and III, are associated with DR3 and DR5 alleles.[13, 14] As regards allergic immune responses to house dust mite (HDM), from the analysis of antigen-specific T-cell clones it has also been established that HLA-DRB1, HLA-DRB3, and HLA-DRB5 gene products (principally DR2, DR5, DR52b, DR51) are all able to restrict the recognition of HDM determinants.[15, 16]

The combined results of these studies, in agreement with many others, indicate that HLA-DR molecules restrict T-cell recognition of the majority of nominal antigens. However, in humans there are two additional families of MHC class II molecules (HLA-DP and HLA-DQ) encoded by the HLA gene complex, both of which may function as restriction elements. As regards HLA-DP, documentation exists that T-cell recognition of viral and alloantigens may be restricted by these class II molecules but may contribute to a minor component of the T-cell repertoire.[17, 18] However, more recently a dominant HLA-DP restricted response to a mycobacterial heat shock protein 65 epitope has been reported in a patient with rheumatoid and arthritis.[19]

The ability to induce nonresponsiveness in allergen-specific T cells offers an approach to the modulation of allergic immune responses, and recently it has been demonstrated that peptide-mediated desensitization of T-cell clones reactive to HDM is accompanied by changes in the cytokine pattern from predominantly interleukin (IL)-4 secretion to that of interferon (IFN)-γ, which may contribute to the downregulation of IgE production.[20] Evidence exists to support that, similar to activation, MHC class II molecules of the appropriate specificity are required for peptide-mediated T-cell anergy,[21] although this analysis is limited to T cells restricted by HLA-DR. Here we extend our findings to report the first isolation of cloned CD4+ T cells that are reactive with a peptide determinant of the group I allergen of *Dermatophagoides pteronyssinus* (*D. pteronyssinus* [*Der p* I]) in the context of HLA-DPB1*0401 class II molecules. This supports the recent population analyses that demonstrate associations between responsiveness to allergens, including HDM, and expression of the HLA-DP phenotype. Furthermore, the results of experiments described here indicate that it is possible to desensitize T cells reactive with HDM and restricted by HLA-DP with specific peptide.

*Nomenclature used is from the WHO Nomenclature Committee. XI International Histocompatibility Workshop, 1991.

MATERIAL AND METHODS

Antigens

Lyophilized extracts of *D. pteronyssinus* and *D. farinae* were the generous gift of Bencard (Brentford, Middlesex, England) and Pharmacia (Uppsala, Sweden). Residues 101-119 (*Der p* I 101-119) of *Der p* I were synthesized by use of standard solid-phase methods, purified by high-performance liquid chromatography (HPLC), and identified by amino acid analysis.[22]

Antibodies

The monoclonal antibodies L243 (anti-HLA-DR)[23] and B7/21 (anti-HLA-DP)[24] were isolated from the culture supernatants of the respective hybridoma cell lines and purified by use of protein A sepharose.

Isolation of human HDM reactive clones

Cloned T cells reactive with *Der p* I 101-119 were isolated from a subject allergic to HDM by limiting dilution cloning, as published elsewhere.[25] In brief, peripheral blood mononuclear cells (PBMCs; 10^6/ml) were stimulated with an optimal concentration of *D. pteronyssinus* (20 μg/ml) for 7 days in RPMI-1640 medium supplemented with 2 μmol/L L-glutamine, 100 IU/ml penicillin/streptomycin (Gibco, Life Technologies, Paisley, Scotland), and 5% screened, inactivated human A^- serum (Blood Transfusion Service, Edgeware England). Lymphoblasts enriched on Ficoll-Paque (Pharmacia) were established as a long-term line in the presence of irradiated autologous PBMCs (5×10^5/ml; 2500 rad), *D. pteronyssinus* (20 μg/ml), and IL-2 (10% vol/vol; Lymphocult T, Biotest Folex, Frankfurt, Germany) and subsequently cloned by limiting dilution. For cloning, viable cells (0.3 cells/well) were resuspended in supplemented medium and plated in Microtest II (Nunclon; Nunc, Roskilde, Denmark) plates together with irradiated autologous PBMCs, *D. pteronyssinus*, and IL-2. After 7 days, growing clones were transferred to flat-bottom 96-well microtiter plates and subsequently transferred to 24-well plates. The clones were maintained with IL-2 every 3 to 4 days and with irradiated autologous PBMCs and *D. pteronyssinus* every 7 days. Before use in assay systems, clones were rested for 7 to 8 days after the last addition of antigen presenting cells (APCs) and antigen.

Proliferation assays

Cloned T cells (10^5 cells/ml) were stimulated with *Der p* I 101-119 (1 μg/ml) or *D. pteronyssinus* (20 μg/ml) in the presence of an equal number of either irradiated autol-

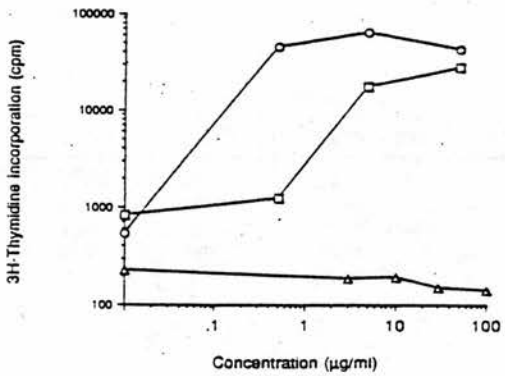

FIG. 1. Antigen specificity of cloned T cells (KS2.12) induced with *D. pteronyssinus*. T cells (10^5/ml) were stimulated with antigen in the presence of irradiated autologous PBMCs as APCs. Proliferation as correlated with [^{3}H]TdR incorporation was measured at 72 hours. Marked dose-dependent stimulation was observed with the peptide epitope *Der p* I 101-119 (○) and with unfractionated *D. pteronyssinus* extract (□). No response was observed to the related HDM species, *D. farinae* (△).

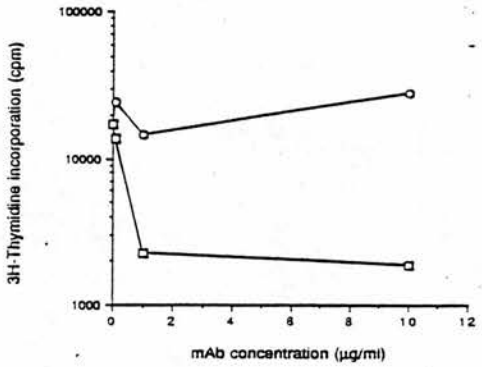

FIG. 2. Anti-HLA-DP class II antibody inhibits the antigen-dependent proliferative response of T-cell clone KS2.12. The blocking effects of murine monoclonal antibodies against human MHC class II molecules were determined in proliferation assays with clone KS2.12 and irradiated autologous PBMCs as accessory cells with *D. pteronyssinus* (20 µg/ml). Marked inhibition was observed with the anti-HLA-DP antibody, B7/21 (□) in contrast to the anti-HLA-DR antibody, L243 (○).

ogous PBMC (2500 rad) Epstein-Barr virus (EBV)–transformed B cells (5000 rad), or mitomycin C-treated murine fibroblasts expressing HLA-D region gene products, as APCs. The murine fibroblasts (DAP3), transfected with DRB1*0101 or DPB1*0401 genes were kindly provided by Dr. J. Trowsdale, (Imperial Cancer Research Fund), Dr. S. Rosen-Bronson, Georgetown University, Washington D.C., and Dr. H. Inoko, Tokai University School of Medicine, Bosheida Isehara Kanagawa, Japan, respectively. In serologic inhibition assays, antibodies were added over a concentration range at the initiation of cultures. After 60 hours of incubation the cultures were pulsed with tritiated methyl thymidine (1 µCi/well, [^{3}H]TdR; Amersham International Inc., Amersham, U.K.) and the cultures harvested 8 to 16 hours later. Proliferation as correlated with [^{3}H]TdR incorporation was measured by liquid scintillation spectroscopy. The results are expressed as mean counts per minute (cpm) for triplicate cultures. The standard error of the mean for all experiments was <20%. The data plotted in each of the figures and tables are from one experiment; however, each experiment was repeated three times or more with consistent results.

Induction of T-cell nonresponsiveness

The induction of peptide-mediated nonresponsiveness was performed as previously reported for T cells restricted by HLA-DR.[26] In brief, cloned T cells (10^6/ml) were incubated with *Der p* I 101-119 (100 µg/ml) for 16 hours in the absence of APCs. Control cultures of cells in medium alone were performed in parallel. The cells were washed extensively after the pretreatment before determining their ability to respond to either an immunogenic challenge of antigen (*D. pteronyssinus* or *Der p* I 101-119) and APCs, or IL-2 or APC alone.

RESULTS

Antigen specificity of T-cell clone

T cells of clone, KS 2.12, in the presence of accessory cells, proliferated in a dose-dependent manner to the inducing antigen, *D. pteronyssinus* whole extract but not a closely related member of the same genus, *D. farinae* (Fig. 1). With use of overlapping synthetic peptides, the reactivity of the T cells was mapped to residues 101-119 of *Der p* I.

Serologic inhibition of the antigen-dependent response of T-cell clone KS2.12

To define initially the subsets of MHC class II molecules acting as restriction elements, monoclonal antibodies directed against framework antigens of HLA-D region molecules were added over a concentration range at the initiation of the proliferation assays containing the cloned T cells (Fig. 2). Maximal inhibition of antigen-dependent proliferation was obtained with anti-HLA-DP antibody (B7/21), whereas the anti-HLA-DR antibody (L243), which recognizes a determinant on DR α chains, failed to modulate the proliferative response. The activity of L243 was confirmed by its ability to block antigen-dependent proliferation of other cloned T cells. The anti-HLA-DP

TABLE I. HLA-D region specificities of the panel of EBV-transformed B cells used as APCs

Cell line	HLA-D specificities		HLA alleles							
			DRB1	DRB3	DRB4	DRB5	DQA1	DQB1	DPA1	DPB1
PGF	DR15 Dw2		1501	—	—	O101	O102	O602	O1	O401
HOM2	DR1 Dw1		O101	—	—	—	O101	O501	ND	O401
YAR	Dr4 Dw10		O402	—	O101	—	O301	O302	O1	O401
SWEIG	DR11(5) Dw5	DR52 Dw25	1101	O202	—	—	O501	O301	ND	O402
AMAI	DR15(2) Dw2		1501	—	—	O101	O102	O602	O1	O402
TOKONAGA	DR15(2) Dw12		1502	—	—	O102	O103	O601	O2	O901
WT24	DR16(2) Dw21		1601	—	—	O201	O102	O502	O1	O301
RML	DR16(2) Dw22		1602	—	—	O202	O501	O301	O1	O402
JVM	DR11(5) Dw'JVM'	DR52 Dw25	1102	O202	—	—	O501	O301	O1	O201
TISI	DR11	DR52 Dw25	1103	O202	—	—	O501	O301	O1	O402
FPF	DR11(5) Dw'FS'	DR52 Dw25	1104	O202	—	—	O103	O6	O1	O201
BM16	DR12(5) Dw'DB6'	DR52 Dw25	1201	O202	—	—	O501	O301	O1	O201
TER-ND	DR103 Dw'BON'		O103	—	—	—	O101	O501	O1	O201, O401
PMG075	DR1 Dw20		O102	—	—	—	O101	O501	O1.O2	O301.O401

ND. Not determined.

TABLE II. The T-cell clone KS2.12 (10^5/ml) was cultured with and without *D. pteronyssinus* in the presence of HLA-D region typed EBV-transformed B cells (10^5/ml) described in Table I, including the autologous EBV-B cells

Cell line	HLA-D specificities		3H-thymidine incorporation (cpm)	
			APC	Dpter (50 μg/ml)
KSE	DR1 DR2		902	35232
PGF	DR15 Dw2		5430	23904
HOM2	DR1 Dw1		2949	46887
YAR	Dr4 Dw10		2911	45938
SWEIG	DR11(5) Dw5	DR52 Dw25	2901	27255
AMAI	DR15(2) Dw2		2352	2880
TOKONAGA	Dr15(2) Dw12		1580	1458
WT24	DR16(2) Dw21		1054	2204
RML	DR16(2) Dw22		2381	23051
JVM	DR11(5) Dw'JVM'	DR52 Dw25	7772	8546
TISI	DR11	DR52 Dw25	1285	22851
FPF	DR11(5) Dw'FS'	DR52 Dw25	1840	1870
BM16	DR12(5) Dw'DB6'	DR52 Dw25	3027	3517
TER-ND	DR103 Dw'BON'		4470	25904
PMG075	DR1 Dw20		854	26182

Dpter: D. pteronyssinus.

Proliferation as correlated with [^{3}H]TdR incorporation measured at 72 hours. Results expressed as mean cpm for triplicate cultures. In both test and control groups SE of the mean <20%.

antibody also gave marked inhibition of a *D. pteronyssinus*-specific T-cell line derived from KS showing that a major component of the T-cell repertoire for *D. pteronyssinus* was restricted by HLA-DP (data not shown).

Restriction pattern of cloned T cells with use of panels of B-cell lines transformed by EBV

On the basis of the serologic inhibition data, the cloned T cells were further examined for responsive-

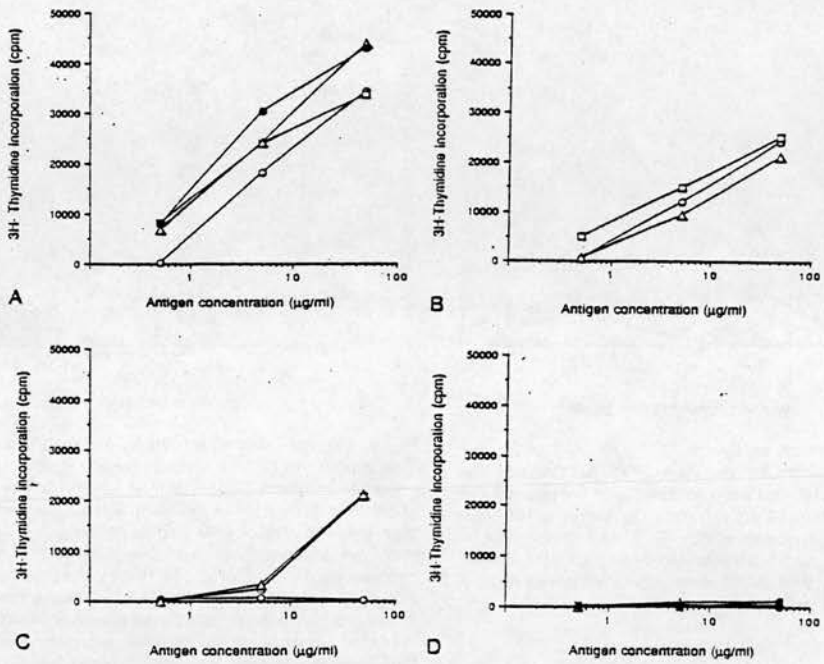

FIG. 3. EBV-transformed B-cell lines expressing HLA-DP class II molecules restrict the recognition of *Der p* I by the T-cell clone KS2.12. Cloned T lymphocytes (KS2.12; 10^5/ml) were cultured with unfractionated *D. pteronyssinus* extract over a concentration range in the presence of an equal number of HLA typed, irradiated EBV-transformed B cells as a source APCs. Strong proliferative responses (**A**) were observed with KSE, the autologous control EBV line (○), PGF (□), YAR (●), and HOM2 (△). Moderate proliferation (**B**) was also induced in the presence of SWEIG (○), PMG075 (□), and TER-ND (△). Weak proliferation (**C**) was observed with RML (□) and TISI (△) at high antigen concentration (50 μg/ml), but AMAI (○) failed to present HDM. No stimulation (**D**) was induced with the cells TOKUNAGA (○), WT24 (□), JVM (△), FPF (●), or BM16 (□).

ness by use of an APC panel consisting of HLA-typed, EBV-transformed, B-cell lines (Table I), including the autologous EBV B-cell line, KSE (DR1, DR2) as a positive control (Table II). A dose-dependent proliferative response was induced to *D. pteronyssinus* when presented by those EBV-B cells homozygous for the DPB1 gene product *0401 (PGF, HOM2, and YAR; Fig. 3, *A*). The B-cell lines PMGO75 and TER-ND, which express the DPB1 gene products *0301, *0401, and *0201, *0401, respectively, also presented antigen to the T-cell clone in a dose-dependent manner (Fig. 3, *B*). The level of proliferation was lower in these cultures than that induced by either the HLA-DPB1*0401 homozygous or autologous B-cell lines. SWEIG, which expresses DPB1*0402, also induced a lower dose-dependent proliferation than that observed with the autologous B-cell line. At supraoptimal antigen concentrations (50 μg/ml), RML and TISI (DPB1*0402) induced marked proliferation. However, the other EBV-B-cell line expressing *0402 (AMAI) failed to present antigen effectively to the cloned T cells (Fig. 3, *C*). Those EBV-B-cell lines expressing the DPB1 products *0201 (JVM, FPF, BM16), *0301 (WT24), and *0901 (TOKUNAGA) failed to induce proliferation (Fig. 3, *D*).

Antigen presentation by murine fibroblasts expressing HLA-DPB1*0401 class II molecules

To confirm the functional role of DPB1*0401 in antigen presentation to T cells of clone KS2.12, murine fibroblasts transfected with the DPB1*0401 gene were used as APCs in proliferation assays with whole *D. pteronyssinus* extract and peptide (*Der p* I 101-

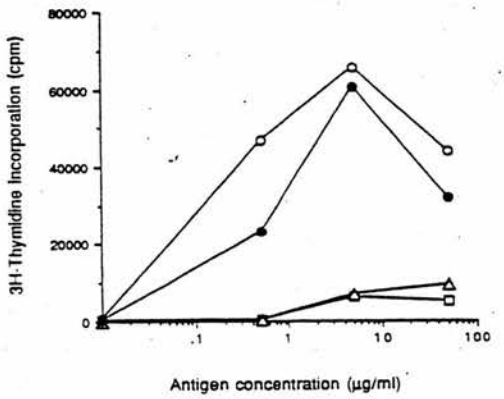

FIG. 4. Recognition of *Der p* I 101-119 by T-cell clone KS2.12 is restricted by the HLA-DPB* 0401 specificity. Cloned T cells (10^5/ml) were cultured with the peptide *Der p* I 101-119 (0.5 to 50 μg/ml) in the presence of different APCs (10^5/ml): autologous EBV-B cells (○) or mitomycin-C–treated murine fibroblasts expressing DPB1*0401 (●), DRB1*0101 (△), and DAP3 (untransfected control murine fibroblast □).

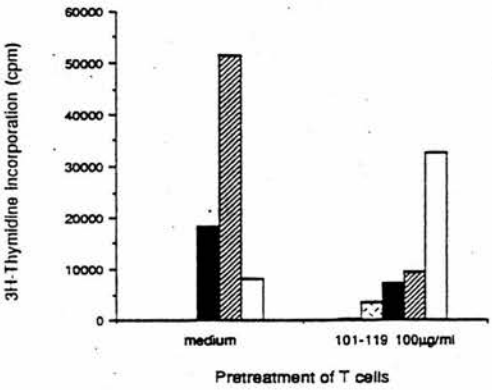

FIG. 5. Peptide-induced inhibition of T-cell proliferation. T cells of clone KS2.12 were cultured for 16 hours in the presence of *Der p* I 101-119 (100 μg/ml) in the absence of accessory cells or in medium alone. The T cells were then washed thoroughly and rechallenged with an immunogenic concentration of *Der p* I 101-119 (1 μg/ml; *hatched histogram*) or crude HDM extract, *D. pteronyssinus* (20 μg/ml; *stippled histogram*) in the presence of irradiated autologous PBMCs as accessory cells, or with medium alone *(solid histogram)*, accessory cells alone *(feathered histogram)*, or with IL-2 *(open histogram)*. Proliferation as correlated with [^{3}H]TdR incorporation was determined at 72 hours. Results are expressed as mean cpm for triplicate cultures. In both test and control groups the SE of the mean was <20%.

119). The transfected fibroblasts induced strong, dose-dependent proliferative responses in the presence of the peptide in a manner analogous to those observed with autologous EBV-transformed B cells as APCs (Fig. 4). In contrast, the nontransfected fibroblast line, DAP3, and a DRB1*0101 transfectant, when used as APCs failed to induce marked antigen-dependent proliferation. Unfractionated HDM extract was only presented to T cells of clone KS2.12 by the DPB1*0401 transfectant at high concentrations (50 μg/ml), and proliferation was decreased compared with presentation by the autologous EBV-B cells (data not shown).

Induction of antigen-specific nonresponsiveness

Preincubation of the HDM-reactive T cells (KS2.12) with specific peptide (*Der p* I 101-119) at 100 μg/ml for 16 hours, in the absence of APCs, induced nonresponsiveness, such that the T cells failed to proliferate when rechallenged with an immunogenic challenge of *D. pteronyssinus* extract or with peptide in the presence of autologous PBMCs (Fig. 5). Responsiveness to exogenous IL-2, however, was enhanced. The presence of anergy was associated with downmodulation of CD3 expression and upregulation of CD25 expression (data not shown), as has been described previously in antigen-induced nonresponsiveness in T-cell clones restricted by HLA-DR.[20]

DISCUSSION

In this report we demonstrate, in the periphery of an atopic individual, the presence of T cells that respond to the group I allergen of *D. pteronyssinus* (residues 101-119) in association with HLA-DP molecules. Furthermore, the results of the experiments reported here indicate that the administration of a supraoptimal concentration of *Der p* I 101-119 is able to functionally inactivate the T cells such that they are unresponsive to an immunogenic challenge of antigen.

The inhibition of antigen-dependent proliferation with anti-HLA-DP, but not anti-HLA-DR framework antibodies, provided the initial evidence that T-cell recognition was restricted by HLA-DP molecules. This contrasts with previous anti-MHC class II antibody blocking studies examining HDM-specific T-cell lines and clones,[15, 27] which suggested that HLA-DR molecules were the major restriction elements. The subsequent detailed analysis of the restriction specificity of HDM-reactive T-cell clones with use of murine fibroblasts expressing the appropriate HLA-D region molecules confirmed that HLA-DRB1, HLA-DRB3, and HLA-DRB5 MHC class II molecules are

functional in the presentation of HDM.[16] The multideterminant nature of HDM may account for this heterogeneity in the HLA class II restriction specificity of the T-cell repertoire reactive with dust mite allergens and, in part, may explain the failure of many genetic epidemiologic studies to establish an HLA linkage. To explore further the HLA-DP restriction of the *Der p* I 101-119 specific T cells, a panel of HLA-typed, EBV-transformed B cells was used as APCs. Antigen-dependent proliferation was observed in the presence of EBV-B cells known to express HLA-DPB1*0401 (PGF, HOM2, YAR, PMG075, and TER-ND). The ability of the DPB1*0402 expressing EBV-B-cell lines SWEIG, RML, and TISI, but not AMAI, to induce proliferation, suggests that different allelic forms of DPB1*0402 may exist with varying structural homology to DPB1*0401. The HLA-DPB1 restriction was confirmed with murine fibroblasts expressing DPB1*0401, which were able to present *Der p* I 101-119 with comparable efficiency to autologous EBV-B cells. In agreement with previous reports examining T-cell recognition of other antigens,[17] our observations demonstrate that HLA-DP molecules are able to restrict T-cell responses to dust mite allergen. It is interesting to note that it has been reported that HLA-DP restricted T-cell clones specific for heat shock protein 65 expressed $CD4^+$, $CD8^-$, TCR *delta* phenotype,[19] whereas the *Der p* I 101-119 reactive T cells described here show positive membrane staining with antibodies specific for TCR-αβ heterodimers. Furthermore, their lymphokine profile was that of the "allergen phenotype,"[28] displaying enhanced mRNA levels for IL-4 and IL-5, but not IL-2 and IFN-γ, which is consistent with the ability to support IgE synthesis (data not shown).

The importance of our findings with respect to the role of HLA-DP molecules in atopy is highlighted by recent epidemiologic studies that suggest positive correlations between specific IgE against common allergens and phenotypic expression of HLA-DP. Using the polymerase chain reaction, Eura et al.[29] observe increases in DPB1*0401 and *0201 frequencies in the response to the group II allergen of *D. farinae*. The findings in this report, based on the class II restriction specificity of cloned T cells reactive with HDM, support these epidemiologic studies. In parallel studies, Young et al.[30] using population analyses report an increase in DPB1*0401 frequencies in atopic individuals, as compared with controls, for a number of important allergens (*Fel d* I; *Can f* I; *Alt a* I; *Phl p* V; *Der p* I, *Der p* II). The expression of DPB1*0401 is 55% in normal individuals, and therefore extension of the population studies combined with the use of cell culture systems is required to dissect the MHC class II restriction of HDM responsiveness. Nevertheless, together these reports suggest a possible important role for HLA-DP as well as HLA-DR gene products in the regulation of allergic immune responses.

The tissue distribution of HLA-DP class II molecules may be as important as their specificity in allergic immune responses. In 5% of adults and 10% of children atopic disease is manifest as extrinsic asthma. Thus increased expression of HLA-D region molecules, including HLA-DP, on lung dendritic cells, the primary APCs in the airway, may have both qualitative and quantitative effects on local immune responses, as has been suggested for dendritic cells isolated from the synovial fluid of patients with inflammatory arthritis.[31] Similarities in the inflammatory components of asthma and inflammatory arthritis imply that studies on the expression of class II molecules on airway APCs may be rewarding.

The ability to render peripheral T-cells nonresponsive to specific antigen forms the theoretic basis of allergen-mediated desensitization. It has been reported that peptides and superantigens of the appropriate specificity are able to induce tolerance and modulate the lymphokine production of T cells reactive to HDM toward that of Th1 type cells.[20] The in vivo potential of peptide-mediated anergy is suggested by the observation that high doses of peptide are able to induce T-cell nonresponsiveness in the presence of APCs.[20] Nevertheless, this analysis has been limited to only those T cells that are restricted by HLA-DR and prompted us to investigate whether or not it was possible to induce tolerance in T cells restricted by HLA-DP molecules. The findings reported here establish that exposure to specific peptide may functionally inactivate T cells restricted by HLA-DP, and that the loss of antigen-dependent proliferation is associated with the transient modulation of the T-cell receptor from the cell surface.[20]

Our findings demonstrate at a clonal level that a component of the T-cell repertoire reactive with HDM, in atopic individuals, may be restricted by HLA-DP class II molecules and support the recent experimental data on HLA associations in allergy that are emerging from the population studies.[29, 30] The ability to functionally inactivate the T cells with use of allergen-derived peptides suggests that such an approach may have therapeutic potential in the management of allergic diseases where T-cell allergen recognition is in the control of HLA-DR and/or HLA-DP molecules.

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Major Histocompatibility Complex Independent Clonal T Cell Anergy by Direct Interaction of *Staphylococcus aureus* Enterotoxin B with the T Cell Antigen Receptor

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Summary

The Staphylococcal enterotoxin superantigens stimulate vigorous responses in T cells bearing certain T cell antigen receptor (TCR) Vβ regions. In addition to activation, these superantigens also impart negative signals to T cells resulting in a profound state of unresponsiveness or anergy. The *Staphylococcus aureus* enterotoxins (SE) B and C_2 bind to a closely related site on major histocompatibility complex (MHC) human leukocyte antigen (HLA)-DR1 molecules. Only SEB, however, interacts with the TCR Vβ3 region of HA1.7, a human HLA-DR1 restricted T cell clone specific for influenza haemagglutinin. In competition experiments, we demonstrated that the induction of anergy in HA1.7 by SEB is unaffected by the presence of SEC_2. These results suggest that SEB-induced anergy is MHC independent and involves a direct interaction between the TCR and SEB. To resolve definitively whether SEB binds directly to T cells in the absence of MHC class II molecules, the cDNAs encoding the HA1.7 TCR were transfected into an MHC class II–negative human T cell line. The addition of SEB to these transfectants resulted in the downregulation of cell surface TCR expression, an increase in the concentration of intracellular calcium ions, the production of lymphokines, and reduced responsiveness to a subsequent challenge with SEB. We conclude that SEB interacts directly with the TCR in the absence of cointeraction with MHC class II molecules, and that this interaction may induce anergy in HA1.7.

A group of exotoxins produced by certain strains of *Staphylococcus aureus* induce vigorous responses in T cells expressing particular TCR Vβ elements (1–4). Such exotoxins have thus been termed superantigens. In common with endogenous murine retroviral superantigens, bacterial superantigens also have the capacity to shape the TCR repertoire by clonal deletion (2, 5) or the induction of anergy (6–8). Since thymic deletion alone fails to explain self-tolerance to antigens expressed exclusively by adults or in sites remote from the neonatal thymus, anergy, which can be induced in mature T cells, is proposed to account for T cell unresponsiveness to these antigens both in in vivo (6–9) and in vitro models (10–12).

Incubation of human T cell clones with supraoptimal concentrations of nominal peptide antigen renders the cells anergic to a subsequent immunogenic challenge (10, 11, 13). In common with antigen-specific T cell activation, antigen-induced anergy is initiated by MHC class II–restricted antigen presentation (14).

More recently, it has been demonstrated that *Staphylococcus aureus* enterotoxin (SE)[1] superantigens, are also able to induce T cell anergy to their native ligand (12, 15). Unlike peptide antigen-induced anergy, however, enterotoxin-induced anergy was not inhibited by anti-MHC class II mAbs (R. E. O'Hehir and J. R. Lamb, unpublished observations).

The aim of the present study was, therefore, to determine whether a Staphylococcal enterotoxin superantigen was able to interact directly with the TCR and induce clonal anergy in the absence of MHC-dependent antigen presentation.

Materials and Methods

Reagents and Cell Lines. HA1.7 is a human $CD4^+$ T cell clone specific for influenza haemagglutinin (HA) peptide 307–319 in the

[1] *Abbreviations used in this paper:* AnPCR, anchored polymerase chain reaction; HA, influenza haemagglutinin; SE, *Staphylococcus aureus* enterotoxin.

context of HLA-DR1. LNAT is an autologous EBV-transformed B cell line used as an APC (16). JRT3T3.5, a CD3-TCR negative, MHC class II–negative mutant of Jurkat (17), was a gift from Dr. A. Weiss (University of California, San Francisco). CTLL-M, a murine IL-2-dependent T cell line used for the bioassay of Jurkat-derived supernatants (18), was obtained from the European Collection of Animal Cell Cultures (EACC) (Porton Down, UK). Oligonucleotides were synthesized by Dr. Ian Goldsmith (Imperial Cancer Research Fund). The HA peptide 307–319 was synthesized by Dr. J. Rothbard (ImmuLogic Pharmaceutical Corp.). FITC-conjugated goat antibodies specific for mouse Igs and anti-CD3 were obtained from Becton Dickinson & Co. (Mountain View, CA). MX6, an anti-TCR Vβ8 mAb was obtained from Dr. S. Carrel (Ludwig Institute, Epalinges, Switzerland). SEB, SEC_1, and SEC_2 were obtained from Sigma Chemical Co., (Poole Dorset, UK) or Toxin Technology, Inc. (Madison, WI).

Cloning of TCR α and β chain cDNA. HA1.7 was purified free of APC and stimulated for 6 h with 1 μg/ml phytohaemagglutinin-P (Sigma Chemical Co.) and 10 ng/ml PMA (Sigma Chemical Co.) to increase TCR α and β chain mRNA expression (19). RNA was purified from 5 × 10^6 cells by the guanidine isothiocyanate-acid phenol method (20). cDNA was synthesized by a standard oligo-dT primed method (21). cDNAs encoding the HA1.7 TCR α and β chains were then amplified using the anchored PCR (AnPCR) (22) as modified by Dr. P. Marche and Dr. O. Acuto (Institut Pasteur, Paris, France).

For amplification of TCR α chain cDNA, an antisense primer was designed to include part of the C region (**bold type**), and EcoRI, and BglII sites: 5′ GCGAATTCAGATCTTAGGCAGA-**CAGACTTGTCACTGG**. 3′; the sense primer for the TCR α chain contained a dC anchor, XhoI, NotI, and SalI sites: 5′ CACTC-GAGCGGCCGCGTCGACCCCCCCCCC 3′. The antisense primer used to amplify the TCR β chain contained part of the C region (**bold type**), KpnI, SalI, and ClaI sites: 5′ GGTACC-GTCGACATCGATCCACCAGCTCAGCTCCACGTGGTCG 3′. The sense primer for the TCR β chain included a dC anchor, SphI, NotI, and SacII sites: 5′ GCATGCGCGCGGCCGCGGA-GGCCCCCCCCCCCCC 3′. 25 cycles of AnPCR were performed, each cycle consisting of 1 min at 94°C, 2 min at 55°C, 3 min at 72°C, and a single final extension of 6 min at 72°C. AnPCR products were isolated, cloned, and sequenced by standard methods. Full α and β coding sequences were constructed using constant region cDNAs derived from the Jurkat TCR α and β chains.

Functional Expression of TCR. The HA1.7 TCR α and β constructs (UBα14/4 and CUBβ1.5) were cloned into the eucaryotic expression vector PJ6Ω, in which cDNA is transcribed under the control of the rat actin promoter (23). pJ6Ω expression vectors containing the hygromycin B (pJ6Ωhygro) or puromycin (pJ6Ωpuro) resistance genes, were made available by Dr. H. Land (Imperial Cancer Research Fund).

The HA1.7 TCR β expression construct (50 μg/ml) was cotransfected with pJ6Ω hygro (25 μg/ml) into 2 × 10^7 JRT3T3.5 by electroporation (450 V at 125 μF). Cells were immediately placed on ice and resuspended in RPMI 1640 containing 10% FCS. After 3 d of culture, the medium was supplemented with 400 μg/ml hygromycin-B (Sigma Chemical Co.).

After 3 wk, the α chain expression construct was cotransfected with pJ6Ω puro into the β chain transfected line by electroporation. Cells were selected in medium containing 400 μg/ml of hygromycin-B, and 4 μg/ml of puromycin (Sigma Chemical Co.), and the resulting cell line assessed for CD3 expression by flow cytometer analysis. Cells were sorted for high CD3 expression three times and then cloned by limiting dilution. Clones were screened for responses to peptide 307–319 and SEB in the presence of LNAT APC. One particularly efficient clone CH7C17 was used for the majority of these studies.

A mock transfected subline designated OGSL1 was also generated. This line underwent all the procedures outlined above, but was electroporated in the absence of plasmid DNA.

Functional Assays. Anergy was induced by incubation of T cells (10^6/ml) with HA peptide 307–319 (up to 100 μg/ml) or SEB (up to 100 μg/ml) for 16 h at 37°C. Cells were thoroughly washed and then challenged with an immunogenic concentration of HA peptide 307–319 or SEB in the presence of irradiated (6,000 rad) LNAT APC. Experiments with HA1.7 were pulsed with 1 μCi of [^{3}H]thymidine (Amersham International, Amersham, Bucks, UK) for the last 16 h of a 72-h culture. For experiments with CH7C17, culture supernatants were taken at 72 h and used to stimulate CTLL-M cells for 24 h. Proliferation was then measured by [^{3}H]thymidine incorporation for the last 8 h of culture.

Assay of Intracellular Calcium. Cells were washed three times with serum-free RPMI, then incubated with 1 μg/ml Fura-2 in RPMI at 37°C for 15 min. Cells were washed twice with HBSS containing 20 mM Hepes at pH 7.3, and resuspended at 10^6/ml. Two ml of cells were placed in a cuvette and warmed to 37°C with stirring in a fluorimeter (The Perkin-Elmer Corp., Norwalk, CT) set to excite at 325 nm and measure fluorescence at 510 nm. The stimulus was added and the fluorescence recorded for 5 min.

Immunofluorescence. Cells were stained for sorting or cell surface phenotype analysis by standard methods using mAb directly conjugated to FITC, or a primary mAb followed by a second layer of FITC-conjugated goat antibodies specific for mouse Ig.

SEB and SEC_2 Competition Binding Assay. SEB was biotinylated by incubating a twofold molar excess of *N*-hydroxysuccinimide active ester of long chain biotin with SEB (1 mg/ml) overnight at 4°C. Unbound biotin was removed by Sephadex G-25 gel filtration. Biotinylated SEB (50 μg/ml) was incubated with 1BW4, an HLA-DR1-Dw1 EBV-transformed B cell line, for 2 h at 4°C in the presence of various concentrations of unlabeled SEB or SEC_2. Cells were washed with PBSA containing 0.1% BSA and incubated at 4°C with FITC-avidin D (Vector Labs., Inc., Burlingame, CA) at 10 μg/ml for 30 min. Cells were washed and resuspended in PBSA containing 0.1% BSA for flow cytometer analysis.

Results

SEC_2 Fails to Compete with SEB during the Induction of Anergy. The Staphylococcal enterotoxins SEC_2 and SEB are known to bind to the same site on MHC class II antigens (24). Unlike SEC_2, however, SEB stimulates T cells expressing TCR Vβ3.1 elements (12). To confirm the identity of the SEB and SEC_2 binding sites on HLA-DR1, biotinylated SEB was incubated with HLA-DR1$^+$ B cells in competition with unlabeled SEB and SEC_2. Consistent with the report that the two enterotoxins bind to a closely related site on the HLA-DR1 molecule, the binding of SEB to HLA-DR1$^+$ B cells was specifically competed with both by unlabeled SEB and SEC_2 (Fig. 1 *A*).

If SEB-induced anergy occurs by a mechanism independent of MHC class II binding, then it should be possible to anergize HA1.7 with SEB after pretreatment with an excess of SEC_2. A 100-fold molar excess of SEC_2 failed to inhibit the induction of anergy in HA1.7 by either SEB or the HA

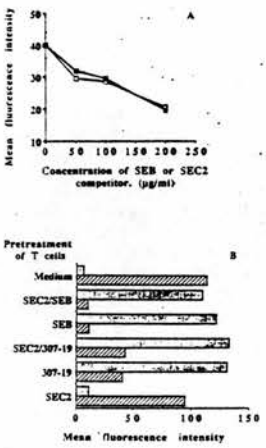

Figure 1. SEC_2 interacts with the SEB binding site on HLA-DR1, but fails to inhibit anergy induction by SEB or HA peptide 307–319 in HA1.7. (*A*). Biotinylated SEB at 50 μg/ml was incubated with IBW4, HLA –DR1-Dw1 EBV–transformed B cells for 2 h at 4°C in the presence of various concentrations of unlabeled SEB (*filled squares*) or SEC_2 (*open squares*). Biotinylated SEB bound to the cell surface was detected using FITC-conjugated avidin and flow cytometer analysis. (*B*) HA1.7 was treated with 50 μg/ml of SEC_2 for 1 h at 37°C then incubated with an anergizing concentration of SEB (0.5 μg/ml) or peptide 307–319 (50 μg/ml) for 16 h at 37°C. Cells were washed and stained with mAb specific for CD3 (*hatched bars*) or CD25 (*shaded bars*).

peptide 307–319 (Fig. 1 *B*). The fall in CD3 expression, and rise in CD25 expression, both characteristics of anergy (12), combined with the failure of HA1.7 to incorporate thymidine after a subsequent immunogenic challenge with HA peptide 307–319 and APC (data not shown), suggested that anergy induction by SEB may not require MHC class II molecules.

Generation of MHC Class II Antigen-negative T Cells Expressing the HA1.7 TCR. The TCR α and β chain cDNAs encoding the HA1.7 TCR were cloned from HA1.7 RNA using AnPCR. 20 clones containing a PCR fragment of the correct size for TCR α were sequenced. All 20 clones consisted of an identical sequence with no evidence for a second TCR α transcript. The HA1.7 Vα region differed from the Vα1.2 subfamily sequence by a single, conservative base change

a HA1.7 TcRα

Vα1.2 — N — JαM
GCT GTG AGT GAG TCT CCA TTT GGA AAT GAG
A V S E S P F G N E

AAA TTA ACC TTT GGG ACT GGA ACA AGA CTC
K L T F G T G T R L
Cα
ACC ATC ATA CCC AAT ATC
T I I P N I

b HA1.7 TcRβ

Vβ3.1 — N — DβI —
TGT GCC AGC AGT TCG ACA GGG TTG CCC TAT
C A S S S T G L P Y

GGC TAC ACC TTC GGT TCG GGG ACC AGG TTA
G Y T F G S G T R L
Jβ1.2 — Cβ
ACC GTT GTA GAG GAC CTG AAC
T V V E D L N

Figure 2. Sequence of HA1.7 TCR α and β chain V-N-(D)-J-C junctional regions. (*a*) TCR α chain. (*b*) TCR β chain. These sequence data are available from EMBL/Genbank/DDBJ under accession numbers X63455 (HA1.7 TCR alpha chain) and X63456 (HA1.7 TCR beta chain).

(25). The N region consisted of twelve nucleotides, and was unique to HA1.7 (Fig. 2 *A*). The core sequence of the HA1.7 Jα region was identical to that used by clone AA13 (26), and was most similar, but not identical, to a member of the M Jα family (27).

20 clones of the single TCR β chain PCR fragment were also sequenced. All 20 clones consisted of a Vβ3.1 region identical to that used by the clone PL4.4 (28), a unique 9-bp N region, and a Dβ region with the conserved core sequence Gly, Leu, Pro (Fig. 2 *B*). The J region was identical to the Jβ1.2 region used by the cell line JM (29).

Full-length coding sequences of the TCR α and β chains were constructed by ligation with the TCR α and β constant regions of Jurkat. Constructs were sequenced to ensure the correct joining of the constant regions. Each TCR cDNA was recloned into expression vectors and transfected into the CD3-TCR negative, MHC class II-negative mutant T cell line JRT3T3.5. The transfected line was cloned by limiting dilution, and the clone CH7C17 was characterized further. A mock transfected line OGSL1 was also characterized in parallel.

The mock transfected subline OGSL1 and the HA1.7 transfectant CH7C17 were examined for surface expression of CD3, TCR-α/β, and TCR Vβ8 by immunofluorescence. OGSL1 was completely CD3, TCR-α/β, and Vβ8 negative, and CH7C17 expressed high levels of CD3 and the TCR-α/β, but no TCR Vβ8, demonstrating that the TCR expressed by CH7C17 was not derived from the native TCR Vβ8 positive Jurkat cell line (data not shown). CH7C17 was also assayed for the expression of MHC antigens. Immunofluorescence analysis using mAbs, specific for monomorphic epitopes of HLA-DR antigens, HLA-DP antigens, all MHC class II antigens (including HLA-DQ), and all HLA class I antigens showed that both the native Jurkat cell line, OGSL1, and CH7C17 expressed no detectable MHC class II antigens (Fig. 3). All cell lines however, were strongly positive for MHC class I antigens (data not shown; Fig. 4 *A*). This confirms results from independent laboratories which demonstrate the

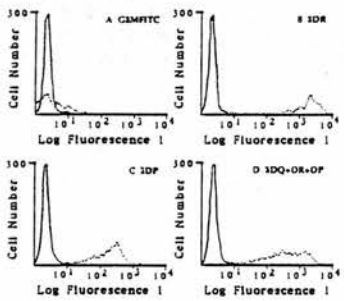

Figure 3. Jurkat transfectants fail to express surface MHC class II antigens. CH7C17 (*solid line*) and LNAT (*dotted line*) were stained with (*A*) goat anti-mouse Igs conjugated with FITC (GaMFITC); (*B*) FITC-conjugated anti-HLA-DR (Becton Dickinson & Co., Mountain View, CA); (*C*) anti-HLA-DP (B7/21/2) and GaMFITC and (*D*) anti-HLA-DQ + DR + DP (CA2.11) + GaMFITC, then analyzed by flow cytometry.

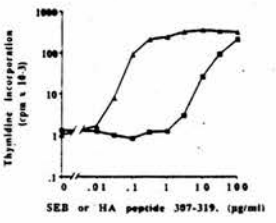

Figure 4. Reconstitution of HA1.7 TCR antigen-MHC specificity. JRT3T3.5 was transfected by electroporation with cDNAs encoding the TCR α and β chain of HA1.7. Transfected cells were cloned (CH7C17) and stimulated with LNAT APC and peptide 307–319 (*filled squares*) or SEB (*filled triangles*). T cell activation was assessed by lymphokine production.

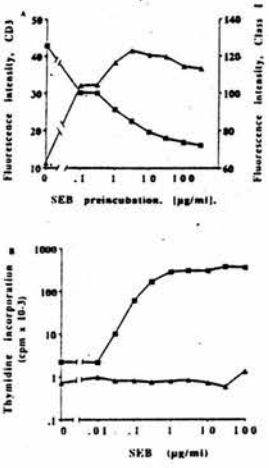

Figure 5. SEB induces downregulation of CD3, and stimulates lymphokine production by CH7-C17 in the absence of MHC class II antigens (*A*) CH7C17 was incubated for 16 h with various concentrations of SEB in the absence of APC; cells were washed and stained with anti-CD3 (*filled squares*, left hand scale) or anti-MHC class I (*filled triangles*, right hand scale) and analyzed by flow cytometry. CH7C17 was stimulated for 16 h with various concentrations of SEB in the absence of APC (*filled squares*); supernatants were taken and assayed for lymphokines. Various concentrations of SEB (*filled triangles*) were incubated for 16 h in the absence of cells. Supernatants were then tested for the direct activation of CTLL-M cells.

complete absence of both cell surface MHC class II antigens on various sublines of Jurkat, (S. Marsh and J. Bodmer, personal communication), and mRNA for HLA-DP, DQ, and DR (45).

Unequivocal evidence that the TCR expressed by CH7C17 was identical to that expressed by HA1.7, was obtained by functional analysis. CH7C17 and OGSL1 were challenged with various concentrations of HA peptide 307–319 and SEB in the presence of LNAT APC. After 24 h of culture, supernatants were assayed for lymphokine content in a CTLL-M bioassay.

As shown in Fig. 4, CH7C17 responded to both HA peptide 307–319 and SEB with a concentration-dependent response, demonstrating that the specificity of the HA1.7 TCR had been reconstituted in CH7C17. The mock transfected cell line, OGSL1, was completely unresponsive to HA peptide 307–319 and SEB presented by LNAT APC. Neither of the cell lines responded to SEC_1 (data not shown).

MHC Class II Independent SEB-mediated Signaling in CH7C17. CH7C17 was incubated in the complete absence of MHC class II-positive APC with SEB at concentrations up to 100 μg/ml. After overnight incubation, cells were washed and assessed for the level of CD3 expression. The culture supernatants were taken for lymphokine assay.

The level of cell surface CD3 on CH7C17 cells decreased in an SEB concentration-dependent manner, such that higher concentrations of SEB resulted in lower cell surface expression of CD3 (Fig. 5 *A*). In contrast to CD3, the level of MHC class I antigen expression was increased with the concentration of SEB (Fig. 5 *A*). MHC class II expression, however, was not induced after incubation with SEB. Using CA2.11, a mAb specific for HLA-DR + DP + DQ antigens, no change was observed in the mean fluorescence intensity of CH7C17 after incubation in medium alone, or in the presence of SEB. A mean fluorescence intensity of 3.4 was observed with CA2.11 and secondary antibody in both the presence and absence of 20 μg/ml SEB. Downregulation of CD3 was specifically due to SEB as SEA, SEC_1, SEC_2, and SEE failed to induce any change in CD3 expression (data not shown). Lymphokine production by CH7C17 cells also increased with the concentration of SEB. This increase was not due to the direct interaction of SEB with the CTLL-M cells (Fig. 5 *B*).

CH7C17 Preincubated with SEB in the Absence of MHC Class II Antigens Fails to Respond to a Subsequent Immunogenic Challenge with SEB. CH7C17 was incubated without MHC class II-positive APC with SEB at various concentrations up to 100 μg/ml. After overnight incubation, cells were washed and loaded with Fura-2 for assay of changes in intracellular calcium ion concentration. Fura-2–loaded cells were then stimulated with SEB (15 μg/ml) in the absence of APC in stirred cultures. Changes in intracellular calcium ion concentration upon stimulation were measured using a fluorimeter.

Within 10 s of restimulation with SEB, the concentration of intracellular calcium increased, peaking at a level dependent upon the concentration of SEB used in the overnight incubation, such that preincubation with higher concentrations of SEB rendered CH7C17 less responsive to the subsequent exposure (Fig. 6). This effect was specific to SEB, as cells incubated overnight with various concentrations of SEC_1 (which does not activate HA1.7) had no effect on the increase in intracellular calcium ions stimulated by 15 μg/ml SEB. Neither SEB nor SEC_1 was able to increase intracellular calcium above basal levels in the TCR-negative cell line OGSL1 (data not shown).

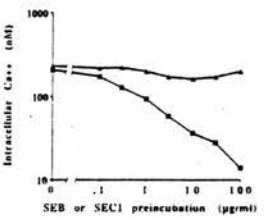

Figure 6. SEB induces unresponsiveness in CH7C17 cells. CH7C17 was incubated for 16 h with various concentrations of SEB (*filled squares*) or SEC_1 (*filled triangles*). Cells were washed and loaded with Fura-2, then stimulated in the absence of APC with 15 μg/ml of SEB. Changes in fluorescence induced by an increase in intracellular calcium ion concentration were measured, and the concentration of intracellular calcium calculated.

Discussion

This study demonstrates that a TCR of defined antigen specificity is able to interact with SEB in the absence of MHC class II antigens. Furthermore, the direct interaction of SEB with the TCR was able to stimulate a program of events leading to clonal anergy in the T cell clone HA1.7.

Characteristically, superantigens are able to bind to all TCRs using particular Vβ regions irrespective of the antigen-MHC specificity of the TCR (3). This implies that the superantigen binding region shared by certain TCR Vβ regions is remote from the CDRs involved in MHC-restricted peptide-antigen recognition. In support of this, two recent studies have analyzed the reactivity of TCR mutants and close Vβ family members with different reactivities, and predicted that superantigen binding sites lie on the side of the TCR molecule, away from the peptide antigen-MHC binding site (30, 31). Similar studies on the MHC requirement of superantigen recognition have shown that many MHC class II antigen isoforms are able to present a particular superantigen to TCR. This suggests that superantigens bind to common sites on MHC class II antigens remote from the peptide antigen binding groove.

The present results demonstrating anergy induction by peptide 307–319 in the presence of SEC_2 (Fig. 1 *B*) support this conclusion. Others have characterized this binding site using cell lines that express MHC molecules with mutations in the peptide antigen binding groove (32), or by demonstrating the binding of labeled superantigens to structurally similar MHC class II molecules (33).

In a recent study of clonal T cell anergy in HA1.7, we demonstrated that human T cells of defined antigen-MHC specificity, when exposed to high concentration of SEB, become anergic to a subsequent immunogenic stimulus with their natural peptide ligand and APC (12). The present report demonstrates that, in contrast to peptide-mediated anergy, SEB-induced anergy in HA1.7 is not MHC dependent. Thus, pretreatment of HA1.7 with anti-MHC class II mAb or SEC_2, which binds to an identical site on HLA-DR1, but fails to interact with the HA1.7 TCR, is unable to inhibit SEB-induced anergy.

To demonstrate directly that SEB interacts with the TCR in the absence of MHC antigens, cDNAs encoding the HA1.7 TCR were introduced into a T cell tumor line which expressed no cell surface TCR and MHC class II. Incubation of these cells with SEB in the absence of APC resulted in SEB concentration-dependent T cell activation, as measured by a rapid increase in the concentration of intracellular calcium ions and lymphokine production. In common with HA1.7, incubation of CH7C17 with SEB in the absence of APC reduced cell surface CD3 expression in a dose-dependent manner. This downregulation was of functional significance, as the cells mounted a reduced Ca^{2+} response to a subsequent exposure to an optimal concentration of SEB. Studies in vivo (34) support the conclusion that the level of TCR-CD3 expression is of fundamental importance in the mechanism of both T cell activation and anergy.

The absence of cell surface MHC class II expression by the transfected mutant cell line derived from Jurkat is of central importance to this study. In addition to immunofluorescence results reported here, (Fig. 3), and those by independent groups (S. Marsh and J. Bodmer, personal communication) analysis of mRNA specific for HLA-DP, DQ, and DR has determined that the parental cell line of CH7C17, Jurkat, does not produce mRNA for HLA-DP, DQ, or DR (45). Furthermore, J. D. Fraser (35), reports that SEA and SEB are unable to bind to the surface of Jurkat cells, confirming that these cells are MHC class II negative. The complete MHC class II negativity of Jurkat was also shown functionally by demonstrating the inability of Jurkat cells to present enterotoxins to unprimed human PBLs (35).

There is a single report that small amounts of MHC class I H chain can be immunoprecipitated from MHC class II–positive cells pulsed with enterotoxin. This study concludes that a small amount of class I H chain might interact with preexisting enterotoxin-MHC class II complexes (35). There is no evidence, however, that MHC class I antigens are able to bind directly to, or present enterotoxins to T cells (36). Indeed, even CD8 positive, class I-restricted CTL TCRs interact with SE only when bound to MHC class II (37). We therefore conclude that SEB-induced downregulation of CD3 in CH7C17 and anergy in HA1.7 is MHC independent and involves a direct interaction between the TCR and SEB.

Other groups have also obtained circumstantial evidence that superantigens might bind directly to the TCR (37–40). Immobilized or cross-linked Streptococcal M toxin has been shown to stimulate native Jurkat cells to produce IL-2 (41). T cells may also be stimulated in the absence of APC by conjugation of a T cell-surface antigen–specific mAb to the same solid support as an enterotoxin (42). There is also a report that enterotoxins may behave in a similar manner to soluble anti-CD3 mAb in their ability to induce early activation events in T cells (38).

The ability to anergize T cells in the absence of MHC class II antigens using enterotoxins has clinical implications in, for example, autoimmune and allergic diseases, where there may be restricted TCR Vβ usage in the harmful T cell responses, but where the antigen is either not determined or extremely complex, and involvement of MHC antigens is ill-defined. In these cases, enterotoxins modified to separate the enterotoxic (emetic) from TCR binding activity (43), and possibly the TCR from MHC binding activity (44), could be used to anergize T cells expressing particular Vβ regions implicated in disease processes.

The authors wish to thank Myriam Ermonval of the Institute Pasteur, Paris, for her advice on the AnPCR; Derek Davies and Andrew Edwards of Imperial Cancer Research Fund (ICRF) for their assistance with flow cytometric analysis and sorting, Susan Lucas and Doreen Cantrell of ICRF for the introduction to calcium assays; Vivienne Weller for her assistance in preparing the manuscript; and Ita Askonas for her helpful comments.

This research was supported by the Imperial Cancer Research Fund, The Medical Research Council of Great Britain, and The Wellcome Trust. R. E. O'Hehir holds a Wellcome Senior Research Fellowship in the Clinical Sciences.

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Received for publication 4 December 1991 and in revised form 12 February 1992.

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Reprinted from Nature, Vol. 303, No. 5918, pp. 625-627, 16 June 1983

Tolerance of T-cell clones is associated with membrane antigen changes

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It is possible to regulate the activity of human influenza virus specific helper T-cell clones either by high concentrations of antigen[1] or by anti-idiotypic suppressor T cells[2]. In the absence of accessory cells, the appropriate peptide antigen recognized by the clones induces specific unresponsiveness. This phenomenon, however, is not the result of cytolysis as responsiveness to IL-2 remained unaltered. This suggests that high-dose immunological tolerance need not involve suppressor T cells, and that peptide antigens can interact directly with the T-cell surface. As recent reports suggest that the T-cell surface antigen T3 is involved in the triggering of T lymphocytes and possibly in antigen recognition[3,4] we have investigated the expression of T3 and other cell surface antigens following the induction of T-cell tolerance. We report here that when a T-cell clone is exposed to a tolerizing concentration of the appropriate peptide antigen, surface T3 antigen is lost in a dose-dependent manner. As loss of surface T3 induced by anti-T3 antibody also results in unresponsiveness to antigen, we conclude that T3 is involved in the process of T-cell triggering by antigen.

The capacity to produce and maintain long-term T-lymphocyte clones has made it possible to analyse T-cell heterogeneity and function with much greater precision than before. We have generated a number of human influenza specific T-cell clones and lines which show diversity of function, such as help[5] suppression[2] and lymphokine production[6]. Some of these have been analysed for the fine specificity of their response to antigen. Clone HA1.7 has been shown to proliferate in culture in response to HLA–DR1 and a 24 amino acid peptide derived from the C-terminal of the HA1 portion of the influenza haemagglutinin (residues 306–329; termed peptide 20)[7].

We have shown that when clone HA1.7 was cultured for several hours at 37 °C with a supraoptimal dose of peptide 20 (p. 20) in the absence of antigen presenting cells, the clone failed to respond by proliferation when subsequently exposed to an immunogenic dose with antigen presenting cells. This unresponsiveness is a form of immunological tolerance and is antigen specific, since the HA1.7 cells which cannot respond to antigen still proliferate in response to optimal concentrations of TCGF. Other T-cell clones in the same culture vessel remain unaffected indicating that this is a specific effect. The cells remain unresponsive for at least one week but during this period are alive and capable of responding to TCGF. These results exclude certain hypotheses for the mechanism of tolerance[1].

Since the T3 antigen is associated with lymphocyte triggering its expression was investigated in clone HA1.7 cultured for 16 h at 37 °C in the absence of feeder cells, or with the relevant influenza A haemagglutinin peptide, p. 20, or an irrelevant haemagglutinin peptide, p. 11. The cells were examined for the expression of T3 antigen by indirect immunofluorescence using a fluorescence activated cell sorter (FACS IV, Becton Dickinson) and for their capacity to respond by proliferation with antigen (p. 20) in the presence of histocompatible irradiated antigen presenting cells (Fig. 1). When cells have been pre-incubated in medium or with p. 11, the majority of the cells are brightly stained by UCHT1 (anti-T3) and far to the right of the scale, but after exposure to p. 20 the fluorescence is much reduced. A dose–response curve with p. 20 shows that the diminution of T3 is dependent on antigen dose, increasing up to the highest concentration used. The phenotypic effects observed following the exposure of HA1.7 to p. 20 are specific in that an irrelevant peptide (p. 11) does not induce the modulation of T3 (Fig. 1). Furthermore, the modulation was not a

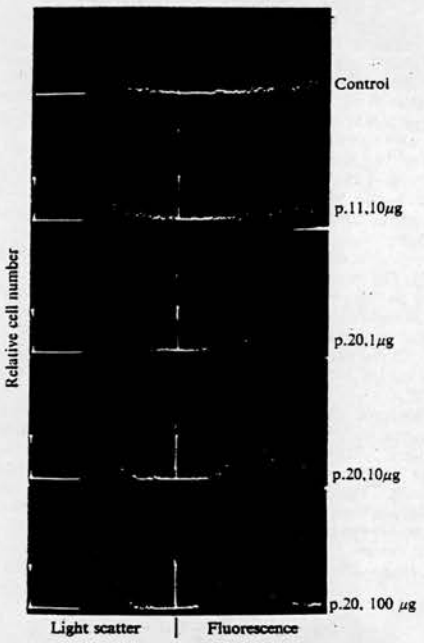

Fig. 1 Indirect immunofluorescent staining with UCHT1 monoclonal antibody of control and peptide antigen modulated clone HA1.7. T lymphocyte clones specific for the synthetic peptides of influenza haemagglutinin[7] were isolated as previously described[1,18]. Briefly, peripheral blood lymphocytes (PBL) were cultured for 6 days with 0.1 μg ml^{-1} of HA (gift of Dr R. G. Webster). The lymphoblasts were enriched on a discontinuous Percoll (Pharmacia) gradient and resuspended in RPMI-1640 (Gibco) containing 10% A+ serum and 20% T-cell growth factor (TCGF), and plated at one cell every third well in Teraski trays with 10^4 irradiated (2,500 rad) autologous PBL and 0.1 μg ml^{-1} HA. After 7 d, growing clones were transferred to 96-well microtitre trays and then to 24-well trays. At each transfer the clones received fresh TCGF and irradiated autologous PBLs together with specific antigen. The clones were expanded in 25 cm^2 tissue culture flasks receiving 20% TCGF every 3–4 d and irradiated autologous PBL and intact virus (A/Texas/1/77) every 7 d. TCGF was prepared from 48 h supernatants of PBL (1×10^6 per ml) cultured with 0.1% purified phytohaemagglutinin (PHA-P; Difco) in complete culture medium containing 1% autologous serum. Before use in tolerance induction or modulation assays, the clones were rested for 6–7 d after the addition of filler cells. Modulation was induced by exposing 2×10^5 clone cells in round bottom microtitre trays in the absence of filler cells for 16 h to HA peptides at the concentrations shown in the figure. In these experiments the peptides used were p. 11 (amino acid residues 105–140) and p. 20 (residues 306–329). Following exposure to peptide or control medium the cells were washed and stained for indirect immunofluorescence with UCHT1 monoclonal antibody and FITC conjugated and immunoabsorbent purified sheep anti-mouse immunoglobulin antiserum[10]. Stained cells were analysed for forward angle light scattering (cell size) and scatter gated fluorescence on a FACS IV.

unique property of either clone HA1.7 or p. 20 in that clone HA2.43, also specific for p. 20, was modulated in a similar manner (Table 1). Secondly clone HA2.61, specific for p. 11 (ref. 1), showed modulation of T3 only after exposure to p. 11 and not p. 20 (Table 1). Exposure of clones HA1.7, HA2.43 and HA2.61 to UCHT1 antibody under similar conditions also leads to a loss of surface T3 antigen (Table 1, Fig. 2) alone, but not other surface markers studied. Preliminary data suggests that significant changes in phenotype and function can be detected after 3 to 4 hours exposure to antigen or UCHT1 antibody. We have not yet investigated in detail the kinetics of re-expression of T3 antigen on modulated cells but other data suggests that modulated TCGF dependent T cells re-express T3 after approximately 48 h[8].

The functional effects of pretreatment with antigen or various antibodies are shown in Table 2. When clone HA1.7 is used, p. 20 but not p. 11 markedly inhibits the response to its specific antigen p. 20, but not to TCGF, indicating that it is an antigen specific effect. UCHT1 causes similar abrogation of the response to antigen. Of the other antibodies, anti-T1 (Leu 1, UCHT2) was weakly inhibitory but not any of the others. It may be relevant that the antigen T1 was partly modulated by p. 20 but not p. 11 (Fig. 2) and can be readily modulated by exposure to anti-T1 antibody (data not shown and reference 8). All the other antibodies shown in Table 2 stained HA1.7 but the level of staining was not decreased by pre-exposure to p. 20.

We have previously shown that exposure of T cell clones to a high dose of peptide antigen in the absence of accessory cells induces a state of specific immunological tolerance implying that T lymphocytes can recognise soluble antigen without processing[1]. The results presented here lend support to this view as, in addition to the functional effects, we have shown that the cells undergo profound phenotypic changes. The data also impiy that the loss of T3 antigen is intimately associated with the loss of responsiveness since both the phenotypic and functional changes can be induced by exposure to antigen or anti-T3 antibody and have the same antigen dosage requirements. It is conceivable, however, that HA1.7 recognizes antigen in association with its own Ia molecules, but fails to proliferate in the absence of other stimulating signals. This question can only be answered by direct binding studies using labelled antigen.

Our results resemble the recent observations of Schlossman and his colleagues who have reported that the T3 antigen of cloned human cytotoxic cells was modulated by exposure to anti-T3 or antibody to clonal specific (clonotypic) molecules[4]. Comodulation suggested that the T3 antigen was closely associated with the clonotypic marker which was considered to be the receptor for antigen. Taken together, both sets of experi-

Table 1 Modulation of T3 antigen in control clones

Clone	Pre-incubation	% Of cells stained with UCHT1 in fluorescence channels		
		1–100	101–200	201–255
HA2.61	p. 11	29	31	40
(anti-p. 11)	p. 20	4	13	83
	UCHT1	63	25	12
HA2.43	p. 11	8	11	81
(anti-p. 20)	p. 20	18	25	57
	UCHT1	53	26	21

T-cell clone 2.61 responds by proliferation to p. 11 and T-cell clone 2.43 to p. 20. Both clones were incubated for 16 h at 37 °C with 20 μg ml^{-1} of p. 11 or p. 20 or 5 μg ml^{-1} of UCHT1 monoclonal antibody. They were then washed and stained with UCHT1 monoclonal antibody for FACS analysis (see Fig. 1). Results are expressed as the percentage of cells (10^3) analysed appearing in fluorescence channels. Cells appearing in channels 1–100 are unstained or very dim, those in channels 101–200 are of intermediate brightness and those in channels 201–255 are brightly stained.

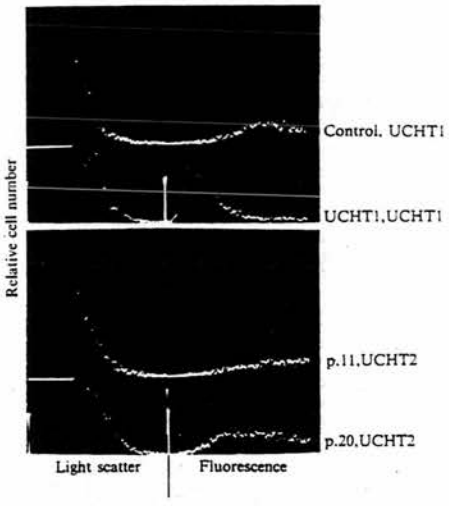

Fig. 2 Modulation of surface antigens by exposure to antibody or peptide antigen. Clone HA1.7 cells were cultured for 16 h in medium alone, in the presence of 5 μg ml^{-1} of purified UCHT1 immunoglobulin or with 20 μg ml^{-1} of p. 11 or p. 20 (see legend to Fig. 1). Following treatment cells were washed and stained with either UCHT1 (anti-T3) or UCHT2 (anti-T1) monoclonal antibodies followed by FITC sheep anti-mouse immunoglobulin antiserum.

Table 2 Effect of monoclonal antibodies reactive with lymphocyte antigens on the induction of antigen specific unresponsiveness

Tolerance induction Antibody (specificity)	Antigen	Response (c.p.m. ± s.e.m.) Antigen	TCGF
—	—	8,821 ± 585	3,981 ± 499
—	p. 20	270 ± 67	4,353 ± 479
UCHT1 (T3)	—	431 ± 70	4,597 ± 412
Leu 1 (T1)	—	6,209 ± 1,251	4,726 ± 442
UCHT1 (T1)	—	4,700 ± 567	4,233 ± 507
9.6 (E receptor)	—	8,271 ± 197	3,968 ± 390
3A1 (most T cells)	—	7,787 ± 1,026	4,593 ± 391
M-T321 (T4)	—	7,885 ± 731	3,951 ± 522
2A1 (HLA Class I)	—	8,995 ± 869	4,871 ± 254
M8 (β2M)	—	9,275 ± 178	4,730 ± 497
Ra1a (HLA Class II)	—	7,291 ± 562	4,103 ± 451
HLe-1 (leukocyte common)	—	6,803 ± 522	4,182 ± 169
—	p. 11	7,984 ± 937	4,144 ± 536

Tolerance was induced as described in detail elsewhere[1]. Cloned T cells (10^5 per ml) were incubated for 16 h with haemagglutinin (HA) peptides or antibodies in the absence of antigen presenting cells. HA peptides were used at 50 μg ml^{-1}, the monoclonal antibodies UCHT1 and Leu 1 at 5 μg ml^{-1} of purified immunoglobulin and other monoclonal antibodies as whole ascites or serum at concentrations at least fivefold greater than the minimum required to give maximal staining by indirect immunofluorescence. After washing, the T cells (2.5×10^4 per ml) were added to irradiated autologous sheep erythrocyte rosette negative (E−) cells (2.5×10^4 per ml) that had been pulsed with antigen. Following 72 h incubation the cultures were pulsed for 8–16 h with 1.0 μCi ^{3}H TdR (Radiochemicals Inc., Amersham) and collected on to glass fibre filters. Proliferation as correlated with ^{3}HTdR incorporation was measured by liquid scintillation spectroscopy. UCHT1 is specific for the T3 antigen present on all mature functional T cells[10], UCHT2 and Leu 1 are against the 67K T1 antigen[11,12], 9.6 binds to the sheep red blood cell receptor[13], 3A1 stains the majority of T cells[14], M-T321 is against the helper/inducer subset (P. Rieber, personal communication), 2A1 is against a non-polymorphic determinant of HLA Class I[15], M8 is against beta-2M[16], anti-HLe-1 is against a common leukocyte antigen[15] and the rabbit anti-Ia is broadly reactive with human Ia-like antigens[17]. HA1.7 expresses the phenotype characteristic of human helper cells (T1, 3, 4 and 11 positive) and is stained by all the reagents listed above.

ments imply that T3 is associated with T-cell antigen recognition. However, certain differences are apparent that influence our hypothesis as to the functional role of T3 and its precise association with the antigen specific T-cell receptor. It has been suggested, based on the comodulation of phenotype and function that T3 represents the constant region of the antigen recognition structure. However, it is unlikely that T3 by itself represents the entire constant region since serological evidence indicates differences between the constant region of the receptors of helper and suppressor T-cell subpopulations[9].

The induction of unresponsiveness by anti-T3 or p. 20 resulted in no enhancement of the IL-2 response, implying that although T3 may be required for antigen-specific signal transmission it is not a prerequisite for IL-2 signal transmission. Furthermore, pretreatment with antigen (or anti-T1) induced phenotypic changes not seen with anti-T3, namely modulation of the T1 antigen. This suggests that although T3 may be linked to the antigen specific receptor it is unlikely that the two are identical since anti-T3 and specific antigen modulate T cells in a different manner. It is our view therefore that T3 is not a structural component of the T-cell receptor but is necessary for transmission of an activation signal following antigen binding. This hypothesis is compatible with all the reported immunological effects of anti-T3.

We are grateful to those who have provided us with monoclonal and other antibodies and to the Department of Haematology, Faculty of Clinical Sciences, University College, London, for permission to use the FACS IV. We thank Dr T. Davies and his colleagues at the National Blood Transfusion Centre, Edgware, for their assistance and Dr R. A. Lerner for helpful discussions. E.D.Z. is supported by the Arthritis and Rheumatism Council.

Received 30 March; accepted 10 May 1983.

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Short paper

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Antigen-specific T cell unresponsiveness in cloned helper T cells mediated via the CD2 or CD3/Ti receptor pathways

We have investigated the role of the CD2 protein in the negative regulation of immune function and report that similar to antigen and anti-CD3, the monoclonal anti-CD2 antibodies ($T11_2$ and $T11_3$) can induce specific unresponsiveness. Antigen and anti-CD2 tolerogenic signals both down-regulated the phenotypic expression of CD3-Ti. In contrast CD2 surface expression was up-regulated after exposure to peptide and down-regulated after anti-$T11_2$ and $T11_3$ preincubation. However, in both instances interleukin-2 receptor surface levels were increased. These phenotypic changes could only be partly explained by variations in the levels of the transcripts encoding the CD3-Ti and CD2 molecules.

1 Introduction

Human T lymphocytes can be activated through either the CD3-Ti receptor complex following co-recognition of antigen and major histocompatibility complex (MHC) gene products [1] or the sheep erythrocyte receptor, the CD2 differentiation antigen [2]. Additionally the CD3-Ti receptor complex can transmit a negative growth or tolerogenic signal, in that the exposure of cloned helper T cells to supraoptimal concentrations of the relevant peptide antigen or anti-CD3 antibody in the absence of accessory cells (AC) can induce unresponsiveness to an immunogenic challenge of specific antigen [3]. This state of anergy results from the modulation of CD3-Ti from the cell surface with reciprocal enhancement of interleukin 2 receptor (IL 2R) and CD2 expression [4]. Therefore, it was of interest to determine if the stimulation of T cells via the "alternative" pathway (CD2 protein) is either able to induce or to reverse antigen-specific T cell unresponsiveness.

2 Materials and methods

2.1 Antigens

Peptide 14 (residues 306–319) and RB6B5 (residues 310–329) of the carboxyl terminus of the HA-1 molecule of influenza virus (H3N2) hemagglutinin (HA) were the gift of Dr. R. A. Lerner, Scripps Research Institute, La Jolla, CA.

2.2 Antibodies and cytofluorometric analysis

Monoclonal anti-CD2 antibodies $T11_1$, $T11_2$ and $T11_3$ were provided by Dr. E. L. Reinherz, Dana-Farber Cancer Institute, Boston, MA. Monoclonal anti-CD3-(Leu 4) and anti-β_2-microglobulin were purchased from Becton Dickinson, Mountain View, CA. Anti-IL 2R antibody (anti-Tac) was the gift of Dr. T. A. Waldmann, NCI, Bethesda, MD. For FACS analysis cloned T cells preincubated with antigen or antibody were washed and stained for indirect immunofluorescence with the monoclonal antibodies (mAb) Leu 4, anti-Tac, $T11_1$, $T11_2$ and $T11_3$ mAb and fluorescein isothiocyanate-conjugated and immunosorbent-purified sheep anti-mouse immunoglobulin antisera as previously described. Stained cells were analyzed on EPICS C (Coulter Electronics, Harpenden, GB). Results are expressed as percentage of cells analyzed appearing in channel no. 1–256.

2.3 Generation of human cloned helper T cells

Cloned helper T cells (HA1.7) reactive with p14 were isolated and characterized as described previously [3]. Briefly, peripheral blood mononuclear leukocytes (PBMC) were cultured for 6 days with HA (0.1 μg/ml). Lymphoblasts isolated on a discontinuous Percoll gradient (Pharmacia, Uppsala, Sweden) were resuspended in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% A^+ serum and IL 2 (20%, Lymphocult T, Biotest Serum Institut, Frankfurt, FRG) and cloned by limiting dilution (0.3 cells/well) in Terasaki trays with irradiated autologous PBMC (10^4/well) and HA (0.1 μg/ml). Clones were expanded in IL 2 every 3–4 days and irradiated histocompatible peripheral blood lymphocytes together with antigen every 7 days. Before use in experiments the clones were rested for 6– days after the last addition of filler cells.

2.4 Induction of T cell unresponsiveness

To induce tolerance, cloned T cells were cultured at 10^6 cells/ml in tubes (Falcon 2058, Oxnard, CA) in the absence of filler cells for 16 h at 37 °C with HA peptides or mAb [3]. Peptides p14 and RB6B5 were used at 50 μg/ml in these experiments. Anti-β_2-M mAb was added at 5 μg/ml and the anti-$T11_2$ and $T11_3$ were added together at a final dilution of 1/200. After washing, the T cells were cultured in proliferation assays.

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Abbreviations: AC: Accessory cells HA: Hemagglutinin IL 2R: Interleukin 2 receptor mAb: Monoclonal antibody(ies) MHC: Major histocompatibility complex PBMC: Peripheral blood mononuclear leukocytes

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2.5. Proliferation assays

Cloned T cells (5×10^4/ml) were cultured together with irradiated histocompatible PBMC (1.25×10^5/ml) and p14 (0.3 µg/ml) or cultured alone with IL 2 in a total volume of 200 µl in 96-well round-bottom microtiter plates. Following 60 h incubation the cultures were pulsed for 8–16 h with 0.1 uCi = 37 kBq of tritiated methyl thymidine ([^{3}H]dThd: Amersham Radiochemicals Inc., Amersham, GB) and harvested onto glass fiber filters. Proliferation as correlated with [^{3}H]dThd incorporation was measured by liquid scintillation spectrometry. The results are expressed as mean counts per minute (cpm) ± % SEM of triplicate cultures.

2.6 Northern hybridization analysis

Total cellular RNA was prepared from cells preincubated overnight in medium or with anti-$T11_2$ and $T11_3$ (anti-CD2), anti-CD3-Sepharose (anti-CD3) or p14. Equal amounts of total RNA (10 µg) were electrophoresed on a 1.1% formaldehyde-agarose gel and transferred to nitrocellulose as described [5]. The filter was sequentially hybridized with ^{32}P-labeled Ti alpha, beta, CD3 delta, CD2 [6] and actin probes as described previously [7]. A different Northern filter was hybridized with the IL 2R probe. This blot was rehybridized with an actin probe (not shown) for quantitation. Several exposures were taken after each hybridization to ensure linearity of response and autoradiographs were quantitated by densitometry. The levels of mRNA were assessed relative to that of actin on the assumption that changes in the actin mRNA level under the different incubation conditions reflected any general variation in mRNA level relative to that of ribosomal RNA. Functional assays on each of the test groups were always performed in parallel.

3 Results and discussion

In this study we have investigated the effects of anti-$T11_2$ and $T11_3$ antibodies on the phenotypic expression and mRNA levels of CD3-Ti, CD2 and IL 2R in the induction of T cell unresponsiveness. For this analysis we have used T cells of clone HA1.7 which are reactive with residues 306–319 of the HA-1 molecule of influenza virus (p14; [3]). These cells when cultured overnight in the absence of AC with p14, but not with an irrelevant HA peptide RB6B5 (residues 310–329), failed to respond to an immunogenic challenge of p14 (Table 1). Similarly, preincubation with anti-$T11_2$ and anti-$T11_3$ antibodies in the absence of AC-induced unresponsiveness to p14, whereas the control antibody anti-β_2-microglobulin had no effect (Table 1). In contrast, both p14 and the combination of anti-$T11_2$ and $T11_3$ enhanced the response to IL 2. In the presence of AC, however, HA1.7 proliferated in response to anti-$T11_2$ and $T11_3$ (Table 1). These results suggest that the CD2 protein can deliver a negative signal that is functionally identical to that of supraoptimal antigen. To determine if both forms of unresponsiveness operate through similar mechanism the cells were firstly examined for CD3-Ti, CD2 and IL 2R expression by indirect immunofluorescence using FACS. In comparison to the medium control, preincubation with p14 or anti-$T11_2$ and $T11_3$ down-regulated CD3-Ti whereas IL 2R expression was enhanced (Table 2 and Fig. 1). However, while expression of CD2 was marginally increased after exposure of the cells to p14, in contrast anti-$T11_2$ and $T11_3$-modulated CD2 from the cell surface. T cell membrane antigen changes following activation with anti-CD3-Sepharose, which mimics the immunogenic effects of antigen/MHC and was included as a control. As observed with the tolerogenic concentrations of p14 and the anti-CD2 antibodies, CD3-Ti was down-regulated and the IL 2R reciprocally up-regulated. There was, however, no change in CD2 expression (Table 2).

Table 1. Anti-CD2 antibody-induced T cell activation and tolerance induction[a]

Tolerance induction T cells.	Antigen/ antibody	Response cpm ± % SEM Antigen/ antibody	AC	IL2
–	–	–	85 ± 9	–
+	–	$T11_2 + T11_3$	8334 ± 12	–
+	–	p14	15140 ± 2	–
+	–	–	–	20771 ± 17
+	p14	p14	117 ± 11	37639 ± 3
+	RB6B5	p14	12876 ± 14	17562 ± 18
+	$T11_2 + T11_3$	p14	141 ± 10	33710 ± 12
+	Anti-β_2-M	p14	17053 ± 1	19770 ± 26

a) Clone HA1.7 (10^6/ml) was incubated with p14 (50 µg/ml), RB6B5 (50 µg/ml), $T11_2$ and $T11_3$ (1/200) or anti-β_2-M (5 µg/ml) in the absence of AC for 16 h. T cells (5×10^4/ml) were then stimulated with irradiated histocompatible PBMC (1.25×10^5/ml) and p14 (0.3 µg/ml) or alone with IL 2. Proliferation was determined by [^{3}H]dThd incorporation. The results are expressed as mean counts per minute (cpm) ± % SEM of triplicate cultures. Control specimens of T cells and PBMC in the absence of antigen, PBMC and T cells alone are shown as is the mitogenic effect of $T11_2$ and $T11_3$ in the presence of PBMC (line 2).

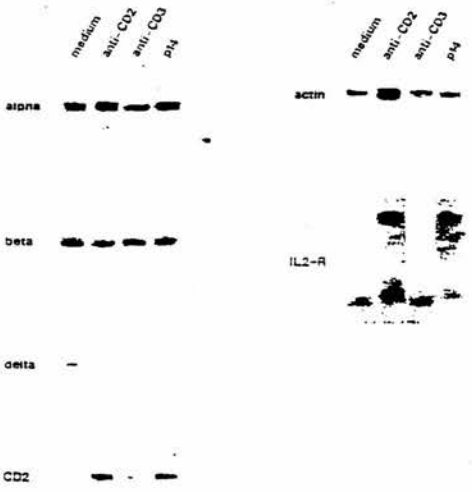

Figure 1. Expression of CD3-Ti, CD2 and IL 2R genes. The incubation conditions used for tolerance or activation and the Northern blotting conditions are as described in Sect. 2.6.

Table 2. Phenotypic modulation of peptide and anti-CD2 antibody-induced T cell unresponsiveness[a]

Tolerance induction:	% of cells stained			
Antigen/antibody	FITC	Anti-CD2	Anti-CD3	Anti-IL2R
Medium	2	71	95	8
p14	12	94	42	12
$TI1_2 + TI1_3$	8	39	9	31
CD3-Sepharose	4	72	76	10

a) Clone HA1.7 (10^6/ml) was pretreated with p14, $TI1_2$ and $TI1_3$, CD3-Sepharose (500 beads/10^6 cells) or in medium alone. The cells were washed, stained and analyzed on FACS as described in Sect. 2.2.

Since p14 and anti-$TI1_2$ and $TI1_3$-induced unresponsiveness was accompanied by phenotypic changes (Table 2 and Fig. 1) the possibility was investigated that these changes were reflected by an alteration in the levels of mRNA encoding the receptor components. When anti-$TI1_2$ and $TI1_3$ was used as a tolerogenic signal Ti beta and CD3 delta mRNAs were reduced by 3–5-fold (compared to the medium control) whereas the level of alpha transcripts remained unchanged although an additional 1.2 kb alpha transcript was observed (Fig. 2). This transcript is most probably a result of transcription from promoter elements upstream of the J alpha elements [8]. In contrast, after preincubation with p14, alpha, beta and delta mRNA levels were not significantly altered although the 1.2-kb alpha species was again induced. CD2 mRNA levels were increased by about 2-fold after preincubation with p14, but were unchanged by anti-$TI1_2$ and $TI1_3$ treatment. In both instances IL2R mRNA levels were increased by at least 10–20-fold. When HA1.7 was activated by an 18-h incubation with anti-CD3-Sepharose Ti alpha and beta, CD2 and IL2R mRNA levels were essentially unaltered whereas CD3 delta mRNA was decreased by about 4-fold. It is emphasized that these changes in transcript levels are relative to the level of actin mRNA and were measured at a single time point. However, changes in the levels of these transcripts may occur at a different time during activation than that which is optimal for the induction of unresponsiveness.

Thus, there was no evidence for a tight coupling of CD3-Ti and CD2 surface expression and steady state RNA levels after the overnight preincubation of HA1.7 with p14 or anti-$TI1_2$ and $TI1_3$. These data do not rule out the possibility that more extensive changes in mRNA levels encoding these surface components occur during shorter incubation periods with p14 or anti-$TI1_2$ and $TI1_3$. They do, however, suggest that the phenotypic changes observed cannot be wholly explained by transcriptional regulation of receptor genes. A translational or, more likely, post-translational control most probably contributes to the cell surface changes. The mechanism of such post-translational control is unclear but may involve the level of intracellular transport, internalization and degradation, or both.

After preincubation with p14 or anti-$TI1_2$ and $TI1_3$, HA1.7 exhibits increased IL2R mRNA and surface expression, a process which is most probably the result of transcriptional control mechanisms. The observed inactivation is, therefore, presumably due to the lack of IL2 expression since the addition of exogenous IL2 results in proliferation (Table 1). Consistent with this observation was the failure to detect IL2 mRNA after the 24-h preincubation of HA1.7 with p14 or $TI1_2$ and $TI1_3$ (data not shown) and that the clone appears to synthesize only low levels of IL2 [9].

This study extends our earlier observations on the tolerogenic effects of antigen via CD3-Ti [3, 4] to the alternative pathway of T cell activation [2] and demonstrates that anti-CD2 antibodies in the absence of AC can induce antigen-specific unresponsiveness through down-regulation of CD3-Ti. In comparable studies it has been reported that activation by CD3-Sepharose is not inhibited by modulation of CD2, whereas preincubation with anti-CD3 antibody did inhibit anti-$TI1_2$ and $TI1_3$ activation although the expression of CD2 was not altered [10]. Other reports suggest that some anti-CD2 antibodies are able to inhibit antigen and mitogen-induced proliferation [11] and that this may operate by suppressing IL2 secretion and receptor expression [12]. In contrast, we and others observed that IL2R expression was enhanced. The implications of these observations for the CD2 and CD3-Ti pathways of activation will most probably require unequivocal elucidation of the physiological role for CD2. This elucidation will be aided by the recent identification of the CD2 ligand as the surface glycoprotein LFA-3 [13]. Various models for the function of CD2 in the thymus have been postulated including a role in inducing the initial expansion of thymocyte immigrants (via IL2R expression) and in the induction of tolerance to self-MHC [10]. In the periphery, a possible role might be a synergistic one, that is, to provide the predominant proliferation signal for a T cell after antigen/MHC recognition via the T cell receptor [14]. Thus, the transduction of both positive and negative signals might be of crucial importance in CD2 function and the results reported here should be interpreted in this context.

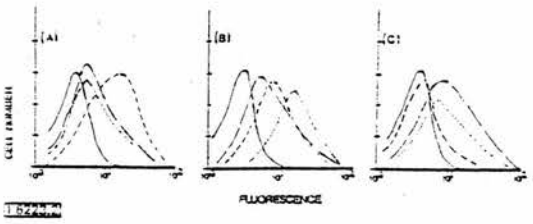

Figure 1. Modulation of CD3 (A), CD2 (B) and IL2R (C) expression after exposure to tolerizing concentrations of the mAb $TI1_2$ and $TI1_3$ (– – –), p14 (· · ·) or medium (—). Control staining with anti-mouse Ig fluorescent antibody (——).

We are grateful to Dr. M. Contreras and colleagues at the National Blood Transfusion Centre, Edgware, for their assistance. Dr. E. Reinherz for donating the anti-$TI1_2$ and anti-$TI1_3$ antibodies. Dr. T. A. Waldmann for donating anti-Tac antibody. Dr. R. A. Lerner for the gift of peptide antigens and Drs. W. Greene and C. Ternorst for the IL2R and CD3 delta probes, respectively. We thank Prof. J. Humphrey and Dr. J. Ivanyi for helpful discussion. Ms. C. Green for FACS analysis and Ms. N. Pinchen for typing the manuscript.

Received May 29, 1987; in revised form August 3, 1987.

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International Immunology, Vol. 3, No. 8, pp. 819–826 0953-8178/91 $3.00

Clonal analysis of differential lymphokine production in peptide and superantigen induced T cell anergy

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Key words: T cells, lymphokines, anergy, superantigen, peptide

Abstract

A failure of T lymphocytes to produce interleukin 2 (IL-2) on restimulation may, in part, account for the specific unresponsiveness that accompanies incomplete activation. The evidence to support this has been derived predominantly from the investigation of the molecular basis of anergy in murine type 1 T cells. In this study, the effects of different tolerogenic signals delivered by specific peptide or *Staphylococcus aureus* enterotoxin on the ability of antigen-specific human T cells to produce lymphokines, both in the induction phase and in established antigen-specific non-responsiveness, have been examined. Although T cell proliferation was decreased by supraoptimal concentrations of specific peptide in the presence or absence of antigen presenting cells, IL-2, IL-4, and interferon gamma (IFN-γ) synthesis were comparable to that of activated T cells. The different tolerogenic signals, all capable of inhibiting proliferation, had selective effects on the secretion of these lymphokines during the induction phase of unresponsiveness. Restimulation of anergic T cells with an antigenic challenge failed to induce lymphokine production, with the exception of allergen-reactive T cells that secreted IFN-γ. This latter observation is relevant to the desensitization of specific responsiveness in allergic disease.

Introduction

The ability to discriminate self from non-self is acquired in the thymus and appears to be mediated both by deletion (1,2) and functional inactivation of self-reactive T cells (3,4). Direct evidence for clonal anergy is derived from the results of recent *in vivo* experiments examining the induction of tolerance to exogenously administered self antigens (5), and the extrathymic expression of non-lymphoid-associated MHC class I and class II molecules in transgenic mice (6,7). These findings parallel those of the previously established human and murine *in vitro* systems of non-responsiveness, which demonstrated that occupancy of the TCR by peptide fragments of antigen bound to class II MHC molecules, in the absence of costimulatory activity, resulted in functional inactivation of the T cells (8–10). Specific non-responsiveness may be achieved by a variety of different stimuli (8–15), including exposure of T cells to free antigen in peptide form (8). Peptides of the appropriate specificity, in the presence of antigen-presenting cells (APCs), were able to inhibit T cell proliferation, although in their absence the concentration of antigen required to achieve a comparable degree of anergy was less (8). Similarly, negative signals delivered by *Staphylococcus aureus* enterotoxins inhibit antigen-dependent but not IL-2-dependent proliferation of T cells expressing particular TCR V_β gene elements (16).

The molecular basis of anergy has been examined predominantly for murine T_h1 type cells, in contrast to the T_h2 cells, that provide B cell help (17), where little is known of the underlying mechanisms (11,18). However, recent analysis of T_h1 and T_h2 cells of the same antigen specificity has indicated that both subsets may be tolerized, although they exhibit distinct differences (19). The failure of T_h1 cells to respond after exposure to antigen presented by chemically modified APCs (9), or by high concentrations of IL-2 (15), was attributed primarily to a lack of IL-2 production, although the activity of other lymphokines was also modulated in the non-responsive T cells (11,12,20,21).

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Transmitting editor: L. Steinman

Received 23 January 1991, *accepted* 22 May 1991

In this report, we have examined the effects of different tolerogenic signals delivered by specific peptide or *S. aureus* enterotoxin on the ability of human CD4+ T cells to proliferate and produce lymphokines. The modulation of lymphokine production was investigated both during the induction phase and in established anergy. In the presence of APCs, although T cell proliferation was decreased by supraoptimal concentrations of specific peptide, the production of interleukin 2 (IL-2), IL-4, and interferon gamma (IFN-γ) was equal to or enhanced as compared with activated T cells. During the induction phase of anergy, different tolerogenic signals that were all capable of inhibiting proliferation had selective effects on the secretion of these lymphokines. Restimulation of the anergic T cells with an antigenic challenge failed to induce lymphokine production, with the exception of allergen-reactive T cells that secreted IFN-γ.

Methods

Antigens

Staphylococcal enterotoxins B, C2, and D (SEB, SEC2, and SED) were purchased from Toxin Technology (Madison, WI). Residues 307 – 319 (HA 307 – 319) of the C-terminus of the HA-1 molecule of influenza virus haemagglutinin and residues 89 – 117 (*p* I 89 – 117) of the group I allergen, *Der p* I, of *Dermatophagoides pteronyssinus* were synthesized using standard solid-phase methods. The peptides were purified by HPLC and identified by amino acid analysis (22). Recombinant *Der p* I was obtained after expression in *Escherichia coli* of the genomic DNA, coding for *Der p* I (a kind gift of Dr W. Thomas, Princess Margaret Children's Hospital, Perth, Australia), as a fusion protein with glutathione transferase (23).

Cloned antigen-specific T lymphocytes

The isolation of cloned T cells reactive with HA 307 – 319 has been reported in detail previously (24). Briefly, peripheral blood mononuclear leukocytes (PBMC) were stimulated with an optimal concentration of purified HA in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 5% screened human AB+ serum. The activated T cells were cloned by limiting dilution in the presence of irradiated autologous PBMC, IL-2 (10% v/v, Lymphocult T, Biotest Folex, Frankfurt, Germany), and antigen. The cloned T cell populations specific for *Der p* I, NP-12 and NP-14, were generated from the blood of a house dust mite allergic patient by stimulating PBMC with a lyophilized extract of *D. pteronyssinus* (1 μg/ml; Diephuis Laboratories, Groningen, The Netherlands) and have been described elsewhere (25). Growing HA-reactive T cells were expanded by stimulation with antigen and filler cells every 7 days. The *Der p* I-specific T cells were cultured and expanded by stimulation with feeder cells every 14 days in the absence of antigen. After stimulation, the T cell clones were expanded with IL-2 every 3 – 4 days. Prior to their use in experiments the T cells were rested, 7 – 8 days for HA1.7 and 10 – 14 days for NP-12 and NP-14, after the last addition of filler cells and antigen.

Induction of T cell anergy

HA-reactive T cells (10^6/ml) were incubated with HA 307 – 319 or the *S. aureus* toxins, and the house dust mite specific T cells with recombinant *Der p* I or *p* I 89 – 117 for 16 h in the absence of APCs. Control cultures of T cells resting in medium or with Concanavalin A (Con A: 30 μg/ml) were performed in parallel. The supernatants were harvested for the determination of lymphokines and the cells then washed extensively before being assayed for their ability to respond to an immunogenic challenge of specific antigen and APCs or IL-2.

Proliferation assays

Cloned T cells (10^5/ml) were stimulated with HA 307 – 319 (1.0 μg/ml) in the presence of mitomycin C-treated murine fibroblasts expressing HLA-DR1Dw1 (10^5/ml; ref. 22) as a source of APCs, or in IL-2 alone. The *Der p* I-specific T cells were stimulated with recombinant *Der p* I or *p* I 86 – 117, in the presence of mitomycin C-treated (50 μg/ml for 45 min), autologous, Epstein – Barr virus (EBV)-transformed B cells as APCs. After 60 h incubation, tritiated methyl thymidine ([^{3}H]TdR; 1 μCi/well; Amersham International, Arlington Heights, IL) was added and the cultures harvested 8 – 16 h later. Proliferation as correlated with [^{3}H]TdR incorporation was measured by liquid scintillation spectroscopy. The results are expressed as mean c.p.m. for triplicate cultures. In all experiments the standard error of the mean was <20%.

Generation of lymphokines

T cells (10^6/ml) were cultured for 24 h with HA 307 – 319 (at 0.5 and 50 μg/ml), SEB, SEC2, or SED (all at 0.5 μg/ml), and recombinant *Der p* I or *p* I 89 – 117 (1 and 100 μg/ml respectively), and the supernatants harvested. Control supernatants from T cells in medium alone or activated with Con A (30 μg/ml) were also assayed for the presence of lymphokines. T cells (10^5/well) exposed to the different pretreatments were restimulated with DR1+ fibroblasts (10^5/well) and HA 307 – 319 (1 μg/ml) in 96-well flat-bottom plates in a total volume of 200 μl. The *Der p* I-specific T cell clones were restimulated with recombinant *Der p* I or *p* I 89 – 117 in the presence of autologous EBV-transformed B cells. Control cultures contained T cells alone, APCs alone, or T cells together with APCs but without added antigen. After 60 h incubation the supernatants were harvested and the levels of lymphokines determined.

Measurement of lymphokines

IL-2 levels were determined using the IL-2-dependent mouse T cell line CTLL-2 (26). IL-4 (27) and IFN-γ (28) were determined by ELISA, as described previously. The assays were performed in duplicate and each experiment was repeated on four separate occasions and yielded similar results. In all experiments the standard error of the mean was <25%.

Fluorescence activated flow cytometry

For flow cytometric analysis, fluorescein-conjugated murine monoclonal antibodies, Leu 5b (anti-CD2), Leu 4 (anti-CD3), Leu 3a (anti-CD4), and anti-IL-2 receptor (anti-CD25), and mouse IgG1 fluorescein isothiocyanate control (anti-KLH) were purchased from Becton Dickinson (Mountain View, CA). T cells were stained directly with saturating concentrations of antibodies. Viable cells, identified by the ability to exclude propidium iodide, were analyzed by flow cytometry using a FACScan (Becton Dickinson). The cell population was analyzed by gating on the volume and light scatter characteristics.

Results

Inhibition of antigen-dependent T cell proliferation and phenotypic modulation after exposure to S. aureus enterotoxin or HA peptide

Preincubation of the HA-reactive T cells (HA1.7) with specific peptide (HA 307 – 319) at 50 μg/ml for 16 h, in the absence of APCs, induced non-responsiveness such that the T cells failed to proliferate when rechallenged with HA 307 – 319 presented by HLA-DR1 + murine fibroblasts (Fig. 1). Lower concentrations of peptide (0.5 μg/ml), that are immunogenic in the presence of APCs, failed to anergize the T cells as compared to the medium control.

After exposure to the appropriate *S. aureus* enterotoxins, SEB and SED, the $V_\beta3$ + T cell clone HA1.7 was also unable to proliferate when restimulated with its natural ligand (Fig. 1). Inhibition of the antigen-dependent response of the T cells induced by both the HA peptide and enterotoxins was associated with enhanced responsiveness to IL-2, thus confirming that the anergy induced was not the result of cytolysis.

T cell non-responsiveness arising from pretreatment with either the *S. aureus* enterotoxins or HA 307 – 319 induced similar changes in the T cell phenotype as determined by flow cytometry (Fig. 2). Down-regulation of membrane cD3 (Fig. 2A) was accompanied by enhanced expression of cD25 (Fig. 2C) as compared to the medium control. No modulation of the T cell phenotype was observed after incubation with SEC2 or HA 307 at 0.5 μg/ml (Fig. 2). Similarly, none of the pretreatments was able to modulate cell surface CD4 (Fig. 2B).

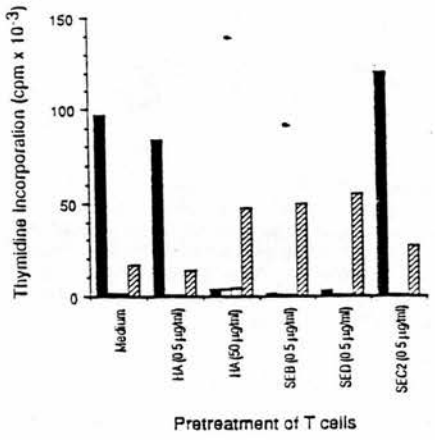

Fig. 1. Peptide and staphylococcal enterotoxin induced inhibition of T cell proliferation. T cells of clone HA1.7 (10^5/ml) were cultured overnight with HA 307 – 319 (0.5 and 50 μg/ml) and SEB, SEC2, or SED (all at 0.5 μg/ml), or in medium alone. The treated T cells were rechallenged with HA 307 – 319 (1.0 μg/ml) in the presence of mitomycin C-treated HLA-DR1Dw1 + murine fibroblasts (solid histogram), DR1 + fibroblasts (open histogram), medium (stippled histogram), or IL-2 (cross-hatched histogram). Proliferation as correlated with [^{3}H]TdR incorporation was determined at 72 h. Results are expressed as mean c.p.m. for triplicate cultures. In both test and control groups the SE of the mean was < 20%.

Lymphokine production following exposure of the HA-reactive T cells to tolerogenic signals

The relationship between lymphokine production and the loss of proliferative response was examined. The HA-reactive T cells were exposed to different immunogenic or tolerogenic signals in the absence of APCs. The supernatants from these 24 h cultures were analyzed for the presence of lymphokines. In parallel, the T cells were assayed for their ability to respond to restimulation with antigen presented by DR1 + -transfected murine fibroblasts, to confirm that anergy had been induced.

Pretreatment of the cloned T cells (HA1.7) overnight with HA 307 – 319 at 50 μg/ml, in the absence of APCs, induced IL-2 production (Fig. 3A), even though at this concentration the peptide rendered the T cells anergic to an immunogenic challenge (Fig. 1). The level of IL-2 produced was greater than or equal to that observed following stimulation with Con A or anti-CD3 antibody and PMA under mitogenic conditions (Fig. 3A). The HA-reactive $V_\beta3$ + T cells failed to produce detectable levels of IL-2 when incubated with SEB or SED under conditions that have been demonstrated to induce non-responsiveness (Fig. 1). Neither HA 307 – 319 at 0.5 μg/ml nor those enterotoxins that interact with TCR V_β gene products of different specificities stimulated measurable IL-2 release (Fig. 3A).

The production of IL-4 by the T cells was determined after identical conditions of activation and tolerance induction as described above. Negative signalling by either the specific peptide or appropriate toxins was accompanied by the induction of IL-4 production, and equalled or exceeded that secreted after stimulation with Con A or anti-CD3 and PMA. Detectable IL-4 production was not observed after incubation of the T cells in the medium or in the presence of the control toxins (Fig. 5B).

Under conditions that inhibit proliferation, the effect of peptide and toxin on IFN-γ production were similar to that observed for IL-4, in that supraoptimal concentrations of specific peptide or staphylococcal enterotoxin induced levels of IFN-γ equal to or greater than that following activation with Con A or PMA and anti-CD3 antibody (Fig. 3C). The relationship between tolerogenic signals and lymphokine production were similar in four separate sets of experiments.

Effect of antigen restimulation on the production of lymphokines by anergic T cells

T cells rendered non-responsive by peptide or enterotoxin were restimulated with HA 307 – 319 in the presence of DR1 + -transfected L cells and the supernatants were harvested to determine if the anergic T cells were still capable of producing lymphokines. Restimulation with an antigenic challenge failed to induce detectable IL-2 production by those T cells that were tolerized by SEB, SED, or HA (Fig. 4A). In contrast, T cells pretreated with the control superantigen SEC2 or the low concentration of HA peptide (0.5 μg/ml) synthesized comparable amounts of IL-2 to the medium control. When rechallenged, the HA-reactive T cells, irrespective of their pretreatment, failed to secrete IL-4 that could be detected in the culture supernatants (Fig. 4B).

Effect of antigen concentration on T cell proliferation and lymphokine production by house dust mite-reactive T cell clones

The *Der p* I allergen-reactive T cell clones, NP-12 and NP-14, proliferated in a dose-dependent manner to the *Der p* I-derived

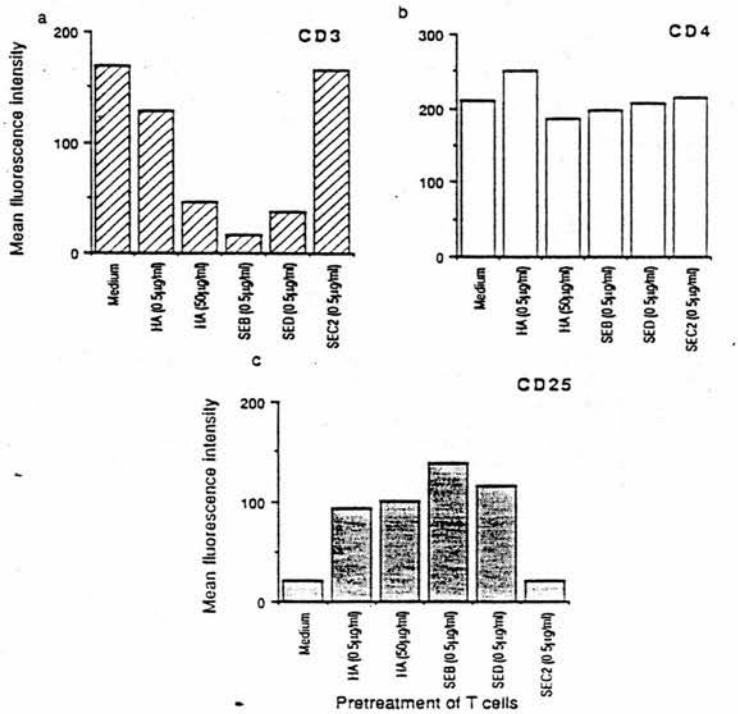

Fig. 2. Phenotypic modulation of HA peptide and staphylococcal enterotoxin anergized T cells. Cloned T cells (HA1.7) were exposed to HA 307 – 319 or the *S. aureus* toxins for 16 h under conditions that induce non-responsiveness. Membrane expression of (A) CD3, (B) CD4, and (C) CD25 was determined by flow cytometry and compared to control cultures of T cells in medium alone or pretreated with a non-tolerizing concentration of the HA peptide.

peptide residues 89 – 117, reaching maximal proliferation at 1 μg/ml (Fig. 5). However, when *p* I 89 – 117 was added at a concentration of 100 μg/ml in the presence of autologous EBV-transformed B cells, the proliferative response of the T cell clones was decreased. In contrast, despite the decreased proliferative response, the T cells in the presence of supraoptimal doses of antigen (100 μg/ml) produced lymphokines in the supernatants. Exposure of the *Der p* I-specific T cell clones NP-12 and NP-14 to tolerizing concentrations of specific peptide or mitogenic concentrations of Con A, in the presence of APCs, induced comparable amounts of IL-2, IL-4, and IFN-γ secretion (Fig. 6). The results presented in Fig. 6 are representative of the patterns of lymphokine production observed in repeated experiments.

Regulation of lymphokine production by house dust mite-reactive T cells in established anergy

The house dust mite-reactive T cell clone NP-14, which produces much greater concentrations of IL-4 under normal immunogenic challenge than the HA-reactive T cells, was no longer able to secrete measurable levels of IL-4 on restimulation of the non-responsive T cells (Fig. 7A). Exposure to low amounts of *p* I 89 – 117 resulted in the production of IL-4, whereas exposure to supraimmunogenic concentrations of peptide followed by rechallenge did not lead to detectable IL-4 release (Fig. 7A). In contrast, the production of IFN-γ was not inhibited by high-dose-specific peptide-induced non-responsiveness of the allergen-reactive T cells (Fig. 7B).

Discussion

The present study examines the differential regulation of lymphokine secretion by human CD3+ T cells during the induction phase of antigen specific anergy and in established T cell non-responsiveness.

The proliferative response of T cells reactive with residues 89 – 117 of the group I allergen of *D. pteronyssinus* (*Der p* I) was markedly decreased by supraoptimal concentrations of antigen, even in the presence of APCs, similar to that described for HA-specific T cells (8). However, in the experiments reported here the lack of proliferation by the *Der p* I-specific T cells was accompanied by secretion of IL-2, IL-4, and IFN-γ in quantities

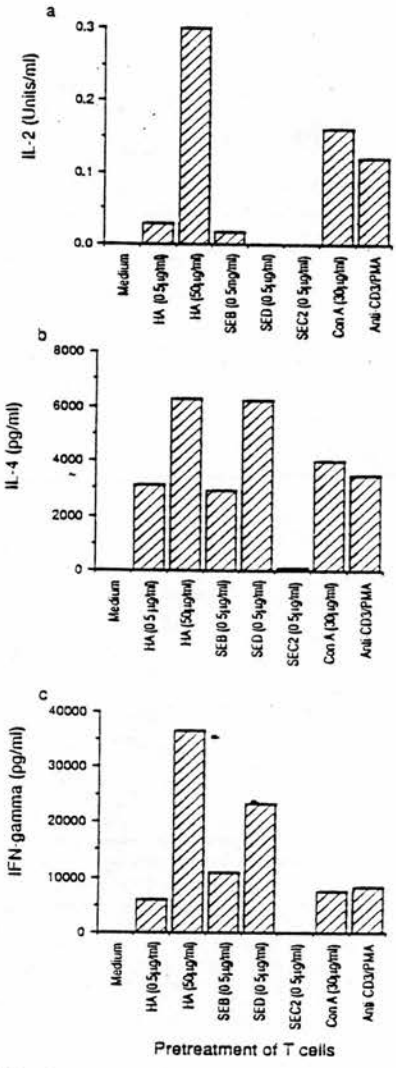

Fig. 3. Effect of activation and the induction of anergy on the production of IL-2, IL-4, and IFN-γ. Cloned T cells of HA1.7 (10^6/ml) were cultured with *S. aureus* toxins, HA peptide, Con A, anti-CD3 antibody, and PMA, or with medium alone. The supernatants were harvested after 24 h and the levels of (A) IL-2, (B) IL-4, and (C) IFN-γ determined as described in Methods. In both test and control groups the SE of the mean was <25%.

which equalled or exceeded that produced during T cell activation. Specialization in function associated with the T_h1 (IL-2+, IFN-γ) and T_h2 (IL-4+) subsets is reflected in their

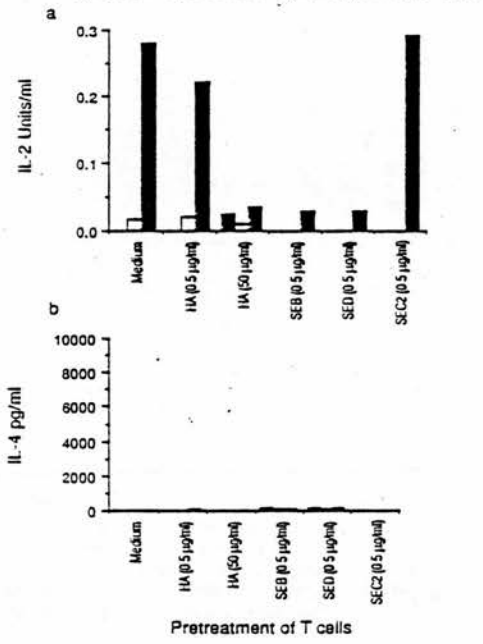

Fig. 4. The failure of anergic T cells to produce lymphokines on restimulation with an immunogenic challenge. Cloned T cells (HA1.7) were cultured with the HA peptide or *S. aureus* toxins under conditions that induce specific anergy. Additional cultures of the T cells in medium or with the HA peptide at a non-tolerizing concentration served as controls. The treated T cells were restimulated with HA 307–319 in the presence of DR1+ transfectants (solid histogram) and DR1+ transfectants (open histogram) or medium alone (stippled histogram) for 72 h. The supernatants from these cultures were assayed for the presence of (A) IL-2 and (B) IL-4. In both test and control groups the SE of the mean was <25%.

lymphokine profiles (29). However, the relationship is not so clearly delineated for human T cells (30,31) and, as described here, the *Der p* I-specific T cells, which are able to support IgE synthesis, also produce IL-2 and IFN-γ (24). However, the amount of IL-4 produced by these CD4+ T cells, in comparison to the HA specific T cells, for example, was elevated. The HA-reactive CD4+ T cells examined here, although fully differentiated and capable of supporting Ig synthesis (8), appeared from their lymphokine profiles to be similar to T_h0 cells (32). The transient inhibition of proliferation in murine T_h1 type cells, mediated by high doses of peptide, resulted from blocking the action but not the synthesis of IL-2 (33). In contrast, analysis of the supernatants of T cells, made unresponsive by antigen presented on chemically modified APCs, revealed that during the induction phase of anergy no IL-2 or IFN-γ was produced (12,34). Therefore, from these different systems, it would appear that antigen delivered in a non-immunogenic form may have opposing effects on the secretion of lymphokines during the

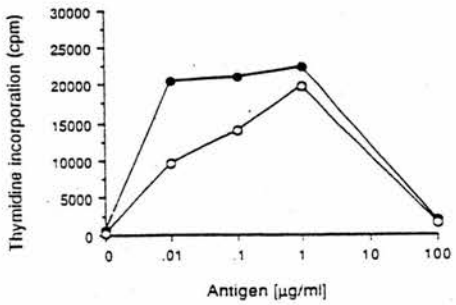

Fig. 5. Inhibition of the proliferative response of house dust mite-specific T cell clones in the presence of high doses of the *Der p* I peptide, *p* I 89 – 117. T cells of clones NP-12 (○) and NP-14 (●) both at 2×10^4/well were stimulated with antigen in the presence of 3.1 autologous mitomycin C-treated EBV-transformed B cells. Proliferation as correlated with [^{3}H]TdR incorporation was determined at 72 h.

induction phase of anergy. The mode of antigen presentation and the signalling requirements of the different T cells investigated may contribute to these differences.

The induction of anergy in human T cells by free peptide is more efficient in the absence of APCs (8) but, as observed in the murine models, non-responsiveness is dependent upon MHC class II molecules, in this case expressed on the membrane of the activated T cells themselves (10). Similar to specific peptide, exposure of T cells with the appropriate TCR V_β gene products to *S. aureus* enterotoxins results in functional inactivation such that they fail to respond to their natural ligand but retain responsiveness to exogenous IL-2 (16,35).

These observations prompted us to investigate the relationship between different tolerogenic signals and the production of lymphokines without the potential influence of other cell types. During the induction phase of peptide-mediated anergy, HA 307 – 319, at tolerizing concentrations, stimulated the production of IL-2, IL-4, and IFN-γ which exceeded that in activation. The effect was similar to that observed for the *Der p* I-specific T cells in the presence of APCs, but contrasted with murine T_h1 cells tolerized by antigen and modified APCs (18). However, murine T cells anergized with immobilized anti-CD3 antibody appeared capable of synthesizing some IL-2 (36). IL-4 and IFN-γ secretion were induced after pretreatment with the enterotoxins SEB and SED under conditions that inhibit antigen-dependent T cell proliferation, whereas there was minimal secretion of IL-2. Despite similar expression of CD25 after exposure to the different tolerogenic signals, it is possible that variation in the level of IL-2 in the supernatants is due to differential consumption by the T cells. The engagement of different regions of the TCR may deliver negative signals that are subtly different in their effects on lymphokine production. The ability of SED to stimulate IL-4 and IFN-γ but not IL-2 production essentially altered the functional phenotype of the T cells. Torbett and Glasebrook (37) observed that the exposure of murine T_h0 cells to fixed APCs inhibited the production of IL-2 but not IL-4. It is not possible from these experiments to resolve whether fixation is influencing APC-derived signals (38) or whether engagement of the TCR is delivering a

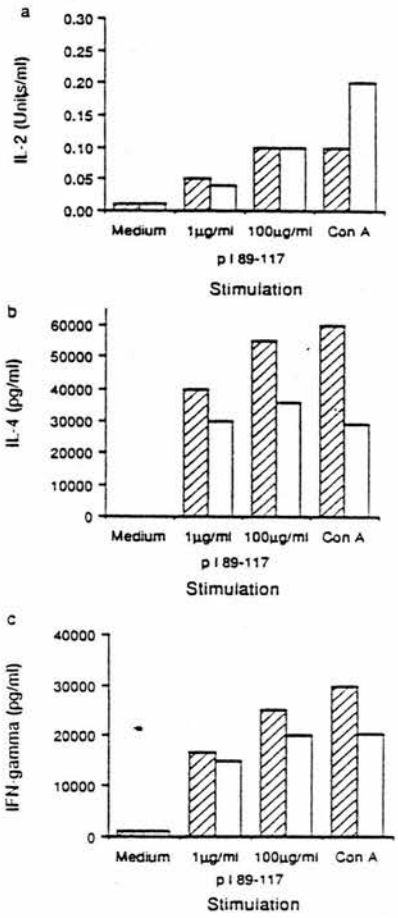

Fig. 6. Lymphokine production by *Der p* I-reactive T cell clones in the presence of high concentrations of specific peptide. Supernatants from 72 h cultures of clones NP-12 (cross-hatched histogram) and NP-14 (open histogram) stimulated with *p* I 89 – 117 (1 or 100 μg/ml), medium, or Con A were assayed for the presence of (A) IL-2, (B) IL-4, and (C) IFN-γ, as described in Methods. In both test and control groups the SE of the mean was < 25%.

negative signal. Comparison of tolerance induction in murine T_h1 and T_h2 cells of the same antigen specificity indicated that proliferation response but not the helper function of the T_h2 subset was resistant to concentrations of antigen capable of tolerizing the T_h1 cells (19). Differences between the human and murine models of *in vitro* T cell anergy are not limited to the effects of negative signalling on lymphokine production. Phenotypic modulation, with the down-regulation of Ti – CD3 (14) and reciprocal enhancement of CD25 (39), occurs during the induction of non-responsiveness in the human T cells, whereas

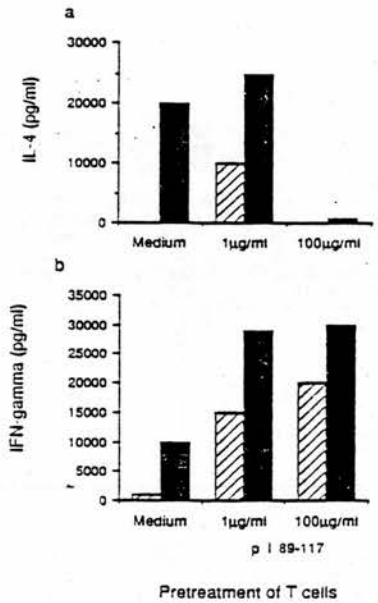

Fig. 7. The differential effects of peptide mediated anergy on IL-4 and IFN-γ production by the *Der p* I-reactive T cells. Cloned T cells (NP-14) after preincubation in medium or specific peptide (1 or 100 μg/ml) were restimulated with *p* I 89–117 in the presence of autologous EBV-transformed B cells (stippled histogram) or EBV-B cells alone (cross hatched histogram) for 72 h. The supernatants from these cultures were assayed for the presence of (a) IL-4 and (b) IFN-γ. In both test and control groups the SE of the mean was <25%.

for murine T cells no alteration in TCR expression is observed between normal and anergic T cells.

T cells tolerized with either specific peptide or toxin failed to recover the capacity to synthesize IL-2 on restimulation with antigen under immunogenic conditions, and supports the finding that murine T cells tolerized by chemically modified APCs or high concentrations of IL-2 (11,21) have reduced transcripts for IL-2 (11,21). The activity of other lymphokines is modulated in anergic T cells; nevertheless, it would appear that IL-2 regulation is critical in the development and reversal (40) of anergy. The failure of T cells from adult mice tolerized to Mls-1 *in vivo* to produce IL-2 on restimulation (5) and the ability of IL-2 to break neonatal tolerance in graft rejection (41) support a central role for IL-2 in activation and non-responsiveness.

Upon rechallenge, IL-4 could not be detected in either the anergized or control cultures of the HA-reactive T cells. As an alternative to IL-4 production being inhibited, these findings may reflect lymphokine consumption by both the activated and anergic T cells. In contrast, only in established non-responsiveness did the *Der p* I-reactive T cells fail to secrete IL-4. There was reciprocal regulation of IFN-γ production, such that the anergic allergen-specific T cells when restimulated with peptide or whole extract of house dust mites secreted IFN-γ in comparable amounts to the normal T cells. IL-4 is required for IgE synthesis by B cells (42), whereas IFN-γ inhibits IgE synthesis (43). Thus, the level of IgE produced is dependent, in part, on the balance between these two lymphokines. Clearly, the ability to selectively modulate lymphokines secreted by the house dust mite-reactive T cells such that IL-4 production is inhibited and IFN-γ is maintained or enhanced would be an advantage in down-regulating the aberrant responses in allergic disease. The findings reported here demonstrate that different signals that are all capable of inhibiting proliferation might have a differential effect on lymphokine production. This information may be relevant in the design of immunomodulatory agents for potential use in the treatment of allergic and autoimmune diseases.

Acknowledgements

This research was supported in part by programme grants from the Wellcome Trust and the Medical Research Council, UK. R.E.O'H. is the recipient of a Wellcome Senior Research Fellowship in the Clinical Sciences. DNAX Research Institute is supported by Schering Plough. This work was carried out in part at ImmuLogic Pharmaceutical Corporation, Palo Alto, California, by R.E.O'H. and J.R.L. as Visiting Scientists.

Abbreviations

APC	antigen-presenting cells
Con A	Concanavalin A
EBV	Epstein–Barr virus
IFN-γ	interferon gamma
IL	interleukin
PBMC	peripheral blood mononuclear cells
TdR	thymidine

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0022-1767/92/1482-0381$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 148, 381–387, No. 2, January 15, 1992
Printed in U.S.A.

UNCOUPLING OF CYTOKINE mRNA EXPRESSION AND PROTEIN SECRETION DURING THE INDUCTION PHASE OF T CELL ANERGY[1]

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The CD4+ T cell clone HA1.7 may be made specifically nonresponsive, or anergic, to its cognate Ag, an influenza hemagglutinin peptide (HA), by pretreatment with the superantigen *Staphylococcus aureus* enterotoxin B or with high concentrations of HA itself. We compare the patterns of mRNA expression and protein production of selected T cell cytokines during the first 24 h after treatments that induce anergy in HA1.7 and during the same period after treatments that simulate normal cellular activation. The cytokines examined include TNF-α, IL-8/neutrophil activating protein-1 and the RANTES/SIS cytokines, a family of small secreted proteins with inflammatory and potential antiproliferative and leukocyte regulating activities. Messenger RNA for TNF-α, human MIP-1α, human MIP-1β, and IL-8 are all induced during the development of clonal anergy in HA1.7, and these levels are significantly higher than those seen during activation of the clone using an anti-CD3 antibody and IL-2. These high levels of mRNA also persist longer than those seen after anti-CD3 and IL-2 activation. However, the increased levels of mRNA are not typically accompanied by increased protein secretion. In all cases but one, the amount of cytokine secreted by HA1.7 cells was greater after anti-CD3 and IL-2 treatments than after anergy- inducing treatments. Thus, the induction of T cell anergy in HA1.7 does not appear to require a general inhibition of T cell cytokine mRNA expression, and, in fact, anergy treatments appear to superinduce certain cytokine transcripts, but anergy-specific posttranscriptional mechanisms may exist by which cytokine release is regulated.

The process of clonal deletion appears to eliminate most T cells reactive to "self" tissues during maturation in the thymus. Nevertheless, additional mechanisms are thought to exist that insure tolerance to host tissues by self reactive T cells in the peripheral circulation. Although these mechanisms are at present largely unknown, experiments in vitro have shown that certain populations of human T cells may be tolerized by supraimmunogenic doses of antigenic peptide such that subsequent exposure to the Ag in the context of MHC does not result in clonal proliferation. IL-2 responsiveness remains intact, however, despite the failure to respond to specific Ag. This type of nonresponsive state, clonal anergy, has been postulated to somehow play a role in extrathymic tolerance of lymphocytes to self molecules in vivo (1–4). Experimental models of nondeletional mechanisms of T cell tolerance established from in vitro systems have been supported by experiments showing the induction of tolerance to exogenously administered self Ag (5) and nonlymphoid-associated MHC class II molecules in transgenic mice (6–8). Inhibition of production of T cell cytokines has been postulated as a mechanism by which clonal anergy is induced (9–11): a lack of production of IL-2 in anergic clones has been reported (9, 10) and nonresponsive T cell clones have also been reported to be poor producers of IL-3 (11).

Activated T cells are known to regulate the transcription of other cytokines including TNF-α, IL-8, and some of the RANTES/SIS[3] cytokines, a newly emerging class of potential leukocyte regulatory factors in the platelet factor 4 superfamily (12–14). RANTES/SIS cytokines are small peptides (M_r 8–16 kDa) secreted primarily by human T cells and monocytes and related by primary structure, including a conserved four cysteine motif (14). This family includes RANTES (15), monocyte chemotactic protein-1 (16–18), I-309 (19), and the human homologs (designated HuMIP-1α, HuMIP-1β) of the murine macrophage inflammatory proteins MIP-1α and MIP-1β (14, 20–23). RANTES/SIS cytokines and their murine counterparts have recently been implicated in a number of important potential immunoregulatory and inflammatory processes (14, 22, 24–26). IL-8 (neutrophil activating protein 1), also a member of the platelet factor 4 superfamily, has been reported to have effects on both neutrophils and T lymphocytes (27–29). To test the ability of the T cell clone HA1.7 to produce these cytokines, and to assess if a general inhibition of T cell cytokine production was a feature of T cell anergy induction, we performed an analysis of the mRNA and protein profiles of selected T cell cytokines in HA1.7 during induction of the nonresponsive state.

MATERIALS AND METHODS

Ag. SEB and SEC2 were purchased from Toxin Technology (Madison, WI). The influenza virus hemagglutinin (residues 307–319) and

Received for publication July 18, 1991.
Accepted for publication October 17, 1991.
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[1] This work was supported in part by a Wellcome Senior Research Fellowship in Clinical Science (R.E.O'H.) and the Medical Research Council, U.K. (J.R.L.). Part of this work was performed while R.E.O'H. and J.R.L. were visiting scientists at ImmuLogic Pharmaceutical Corporation, 855 California Avenue, Palo Alto, CA 94304.
[2] Adress correspondence and reprint requests to Dr. Thomas J. Schall, Genentech, Inc. 460 Point San Bruno Blvd., South San Francisco, CA 94080.

[3] Abbreviations used in this paper: SIS, small, inducible, secreted; NAP-1, neutrophil-activating protein-1; HA, hemagglutinin; SEB, *Staphylococcus aureus* enterotoxin B; SEC2, *S. aureus* enterotoxin C2; MIP, macrophage inflammatory protein; HDM: House dust mite.

HDM (*Der p* II: residues 36–60) peptides were synthesized using standard solid-phase methods on an Applied Biosystems 430A synthesizer (Foster City, CA) purified by reversed phase HPLC and analyzed by amino acids analysis as previously described (30).

Antibodies. For flow cytometric analysis fluorescein conjugated murine monoclonal antibodies, anti-Leu 5a (CD2), anti-Leu 4 (CD3), anti-Leu 3a (CD4), anti-IL-2R (CD25), and mouse IgG_1 FITC control were purchased from Becton Dickinson (Mountain View, CA).

Cloned HA reactive T lymphocytes. The isolation and characterization of the cloned human T cells reactive HA 307–319 have been reported elsewhere (31). Briefly, T cells activated with immunochemically purified HA were resuspended in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 100 U/ml penicillin, 100 ml/ml streptomycin, 2 mM L-glutamine, and 5% screened human AB^- serum and cloned by limiting dilution in the presence of autologous irradiated PBMC, IL-2 (Lymphocult T, Biotest Foiex, Frank, FRG), and Ag. Growing T cells were expanded by cyclic stimulation with Ag and filler cells every 7 days and IL-2 every 3 to 4 days. Before their use in experiments the T cells were rested for 7 days after the last exposure to Ag and filler cells.

Induction of T cell non responsiveness. T cells (10^6/ml) were incubated for 16 h with the *Staphylococcus aureus* toxins (0.5 mg/ml). Control cultures of T cells in medium or activated with insolubilized anti-CD3 antibody (12 mg/ml) and IL-2 were examined in parallel. The cells were washed extensively after the pretreatment before determining their ability to respond to either an immunogenic challenge of Ag (HA 307–319) and APC or IL-2.

Proliferation assays. Cloned T cells (10^5/ml) were stimulated with HA 307–319 (1.0 mg/ml) or the *S. aureus* toxins at concentrations indicated in the figures, in the presence of mitomycin C-treated murine fibroblasts expressing HLA-DR1 (10^5/ml) (31) as a source of APC, or in IL-2 alone. After 60 h of incubation, [^{3}H]TdR (1 mCi/ml; Amersham International, Arlington Heights, IL) was added and the cultures harvested onto glass fibre filters 8 to 16 h later. Proliferation as correlated with [^{3}H]TdR incorporation was measured by liquid scintillation spectroscopy. The results are expressed as mean cpm for triplicate cultures. In all cases the SE of the mean was <20%.

Fluorescence flow cytometry. T cells were stained directly with saturating concentrations of fluorescein-conjugated murine mAb, anti-Leu 5a (CD2), anti-Leu 4 (CD3), anti-Leu 3a (CD4), anti-IL-2R (CD25) using a mouse IgG_1 FITC control. Viable cells, identified by their ability to exclude propidium iodide, were analyzed by flow cytometry using a FACScan (Becton Dickinson). The cell population was analyzed by gating on the volume and light scatter characteristics.

Northern blot analysis: solid phase ELISA. RNA was extracted at 0, 2, 4, and 24 h after treatment described above from 5 million cells/time point using the method of Chomzynski and Sacchi (32). A total of 10 μg of cytoplasmic RNA per time point was fractionated on a 1% agarose/formaldehyde gel and transferred to a nylon membrane (Genescreen, Du Pont, Wilmington, DE) per the manufacturer's recommendation. Probes representing the coding regions of the cDNA listed in Figure 3 were labeled with ^{32}P-dATP using the random hexamer method, and applied to the blot in 50% Formamide hybridization solution at 42°C for 16 h per the membrane manufacturer's recommendation. Blots were washed in 0.2X SSC; 1.0% SDS at 55°C and exposed at -70°C on Kodak XAR5 film. The same blot was serially hybridized with each probe listed. Probes were stripped by boiling the blot in water for 5 min before subsequent hybridization. Densitometric quantitation of Northern blots was performed using a laser densitometer manufactured by Molecular Devices Corporation (Menlo Park, CA) using the Image Quant 3.0 software package. IL-8 and TNF concentrations in HA1.7 supernatants were assayed by solid phase ELISA by the Genentech Immunology Research and Assay Technology section; the results shown represent mean values of two assays each comprised of triplicate determinations for each data point. In all cases the SE of the mean was less than 10%.

Western blot analysis. Antisera for Western analysis were generated by immunizing female NZW rabbits with the synthetic peptides CYTO 1 (PMGSDPPTAC) and CYTO 3 (CADPSEEWVQKY-VSDLELSA) representing the amino terminus of HuMIP-1β and the carboxy terminus of HuMIP-1α, respectively, which were conjugated to the carrier protein soybean trypsin inhibitor via their cysteine residues. Preimmune sera were collected from rabbits before immunization. Immunologic blot analysis were performed as described (33) with both antisera (each diluted 1/2000) combined to detect HuMIP-1α and HuMIP-1β proteins on the same blot. Experiments with recombinant HuMIP-1 proteins have shown that HuMIP-1α migrates as a lower band relative to HuMIP-1β due to presumed glycosylation differences (14). Background staining was assessed by incubating a duplicate blot in pooled preimmune serum.

RESULTS

HA1.7 is a $CD4^+$ T cell clone that recognizes the carboxyl-terminus of influenza virus HA (peptide HA; residues 307–319) (30, 31). To test its cytokine mRNA and protein production profiles during tolerance induction, HA1.7 was tolerized to this Ag by preincubation for 16 h with high doses of HA or by exposure to the superantigen SEB in the absence of APC (Fig. 1) (34). Cells so pretreated are unable to proliferate, as measured by [^{3}H]TdR incorporation, to subsequent immunogenic challenge of HA presented by murine fibroblasts expressing HLA-DR1, but retain their ability to proliferate in response to IL-2 (Fig. 1). Cells pretreated with control conditions medium alone, anti-CD3 mAb, anti-CD3 mAb plus IL-2, SEC2, or a HDM peptide proliferate well to both subsequent challenge with HA plus APCs and to IL-2 stimulation (Fig. 1). No proliferation is seen under any of the conditions to APC alone. Induction of tolerance in response to HA or SEB is accompanied by cell surface phenotypic changes that mimic those seen with T cell activation (Fig. 2). Analysis of cell surface markers by flow cytometry indicates an increase in the levels of CD2 and CD25 in response to HA, SEB, and anti-CD3 antibodies alone or with IL-2, but a reduction of CD3 by the same treatments (Fig. 2). These cell surface molecules are unaffected by the control conditions, and levels of CD4 are not significantly affected by any of the treatments (Fig. 2).

Northern blot analysis of RNA extracted from HA1.7 cells after various treatments reveals little mRNA for most of the cytokines tested in untreated cells but a striking induction and accumulation of mRNA for many of these molecules after the HA or SEB tolerizing treatment. Only RANTES mRNA is present in untreated HA1.7 cells, and its levels are not greatly changed by

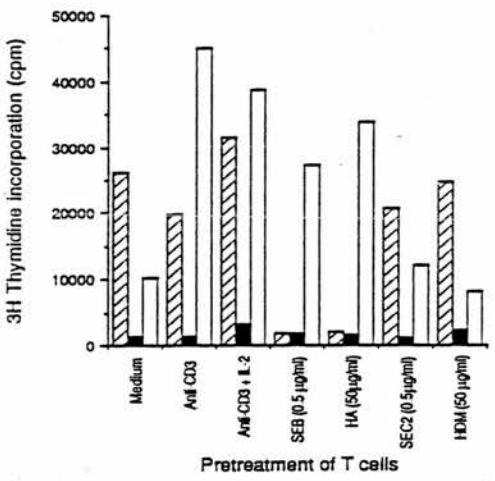


Figure 1. Induction of T cell anergy in the T cell clone HA1.7 after exposure to SEB or HA peptide. T cells were exposed to SEB or HA 307–319 under conditions which induce nonresponsiveness (34). Control T cells populations were pretreated by culturing in medium alone, anti-CD3 antibody alone and in combination with IL-2, SEC2, or HDM. After pretreatment the T cells were assayed for their ability to respond to restimulation with HA 307–319 and accessory cells (mitomycin C-treated murine fibroblasts expressing HLA-DR1; *stripped bars*), accessory cells alone (*solid bars*), or IL-2 (*open bars*). Proliferation as correlated with [^{3}H]TdR incorporation was determined at 72 h.

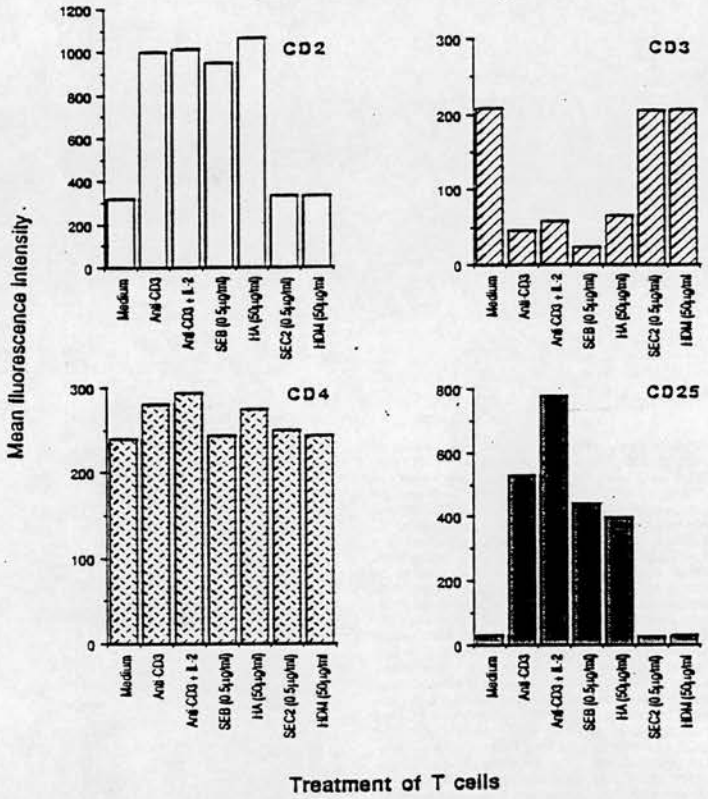
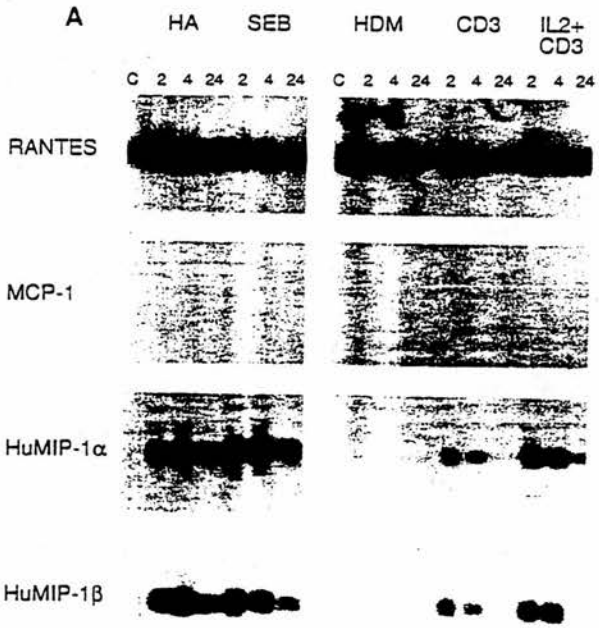
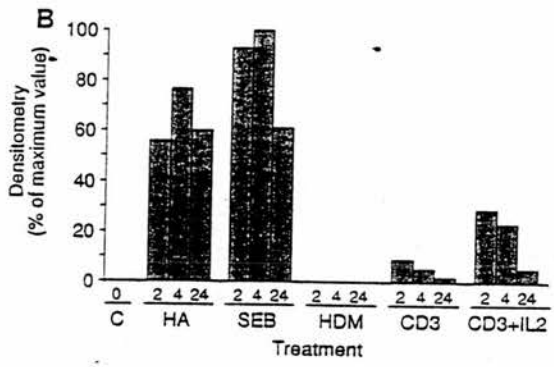


Figure 2. Phenotypic modulation of T cells anergized with SEB or HA peptide. Membrane expression of CD2, CD3, CD4, and CD25 of the anergic T cells was examined by flow cytometry and compared to control cultures or those activated with anti-CD3 and IL-2. Phenotypic changes in response to tolerizing treatments mimic those of activation treatments. CD4 levels are unchanged.

either tolerizing (HA or SEB) or activation (anti-CD3 antibody with or without IL-2) treatments (Fig. 3*A*). This is in contrast to observations in other T cell populations, where levels of RANTES mRNA are markedly reduced after activation via the TCR (15). We do not know if the lack of reduction of RANTES mRNA in these cells after stimulation is a specific property of tolerizable T cells. In contrast to RANTES, monocyte chemotactic protein mRNA is not detected in the HA1.7 clone (Fig. 3*A*). The HuMIP-1 genes, however, typify the pattern of inducible cytokine mRNA investigated in this T cell clone. HuMIP-1α mRNA accumulates dramatically after tolerizing treatments (Fig. 3*A*), and these levels of HuMIP-1α mRNA are higher, and persist longer, than levels induced by anti-CD3 activation in the presence or absence of IL-2 (Fig. 3*A*). Laser densitometry measurements (Fig. 3*B*) reveal that peak levels of HuMIP-1α mRNA expression after anti-CD3 stimulation are at least 10-fold less than peak levels seen after SEB treatment of HA1.7. The expression of the mRNA for HuMIP-1β, a cytokine nearly 70% identical to HuMIP-1α in primary structure, is similar to that seen with HuMIP-1α (Fig. 3*A*) except the levels of HuMIP-1β RNA are not as abundant and do not persist as long as HuMIP-1α mRNA. Neither HuMIP-1α nor HuMIP-1β are appreciably induced by treatment with HDM peptide (Fig. 3*A*) or with SEC2 (data not shown), indicating that HuMIP-1 mRNA induction in HA1.7 cells is specific to activation or tolerizing signals. Thus, although related in primary structure and clustered on human chromosome 17 (35, 36), the RANTES/SIS cytokines are differentially transcriptionally regulated in HA1.7 during cellular activation and during the induction of clonal anergy.

To assess whether the release of HuMIP-1 proteins into HA1.7 culture supernatants correlated with mRNA levels during anergy induction and activation treatments, Western blot analysis was performed using antipeptide antisera that would discriminate between HuMIP-1α and HuMIP-1β. Although there is significantly greater mRNA accumulation during anergy induction than during anti-CD3 antibody treatment in the presence or absence of IL-2 (Figs. 3, *A* and *B*), the amount of HuMIP-1α and -1β protein secreted does not necessarily mirror the mRNA pattern (Fig. 3*C*). For example, SEB anergy induction treatments result in the highest levels of HuMIP-1α mRNA in HA1.7 (Figs. 3, *A* and *B*), but the secretion of HuMIP-1 proteins from SEB-treated cells appears no greater than that detected from anti-CD3-activated cells (Fig. 3*C*), which express less than one-tenth the mRNA. Moreover, even though the greatest amount of protein is detected in supernatants collected from cells 24 h after treatment with HA peptide, corresponding with the fact that these cells contain high levels of both HuMIP-1α and -1β mRNA, titration experiments reveal that the level of protein secretion is only two to three times that of the





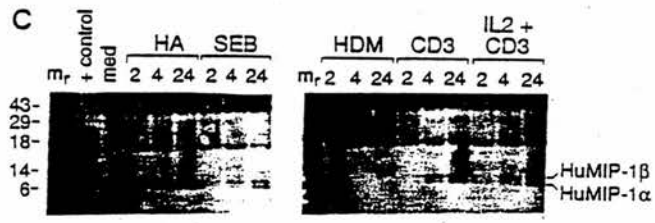


Figure 3. *A.* Northern blot analysis of RANTES/SIS cytokine expression in HA1.7. HA1.7 cells were treated as in Figure 2 and cytoplasmic RNA was harvested after 2, 4, and 24 h incubation for each treatment. RNA from untreated control cells is in lanes designated *C*. Ethidium bromide staining and approximately equivalent intensities with an actin probe confirmed equal amounts of RNA were loaded in each lane (not shown). The same blot was stripped and reprobed with a labeled cDNA fragment representing the coding region of each cytokine listed in the *left margin*. *B*, Laser densitometry quantitation of HuMIP-1α RNA. *C*, Western blot analysis of HuMIP-1α and -1β proteins in HA1.7 cell supernatants after activation and anergy induction treatments. Supernatants, collected from cells treated as described above, were fractionated on a 10 to 20% tricene-Tris SDS-PAGE and immunoblotted. Plus (+) lanes represent positive control sample consisting of recombinant HuMIP-1α. M_r lane is migration of M_r markers (BRL prestained low m.w. standards) in thousands.

anti-CD3 treated cells, which express considerably less HuMIP-1 mRNA. Thus, the secretion of the HuMIP-1 cytokines appears to be posttranscriptionally regulated during anergy induction. In addition, posttranscriptional regulation mechanisms may differentially control the secretion of HuMIP-1α and -1β during Ag-specific anergy induction (HA treatment) as compared to superantigen-induced anergy (SEB treatment).

Posttranscriptional regulation may be controlling the secretion of RANTES protein as well in HA1.7. We were also unable to detect RANTES, as assayed by Western blot, in the supernatants of these cells, although abundant mRNA levels exist.

IL-8/neutrophil-activating protein-1 is related to the

RANTES/SIS cytokines by a modification of the four cysteine structural motif (13, 14, 27, 28). As with HuMIP-1α and HuMIP-1β, IL-8 mRNA is detected in HA1.7 during cellular activation and the induction of anergy. Treatment of HA1.7 with anti-CD3 and anti-CD3 plus IL-2 results in detectable levels of IL-8 mRNA, and higher levels are seen after HA and SEB anergy treatment (Fig. 4, *top panel*). No IL-8 mRNA is seen after SEC2 or HDM treatment. However, although mRNA levels appear higher in response to the tolerance inducing signals than the anti-CD3 activation signals, this does not correlate with the amount of IL-8 protein secreted into the cell culture medium. A solid phase ELISA using two anti-IL-8 mAb reveals concentrations of secreted IL-8 are markedly higher with anti-CD3 + IL-2 treatment (~55 ng/ml after 24 h) (Fig. 4, *lower panel*), with levels present after HA, SEB, and anti-CD3 alone being approximately 10-fold less (~5 ng/ml after 24 h of HA treatment) (Fig. 4). No IL-8 was detected in the supernatants of unstimulated cells or cells treated with SEC2 or HDM. Thus, although HA1.7 cells express IL-8 mRNA and release IL-8 protein specifically in response to activation or tolerizing treatments, protein secretion appears to be posttranscriptionally regulated in anergized cells. The production of IL-8 by HA1.7 is consistent with the observation that these cells produce a neutrophil chemotactic activity (37), and demonstrates that at least some T cells have the capability to actively secrete IL-8.

The expression of TNF-α mRNA and secretion of this cytokine was also examined, and the pattern found mimics that seen with the HuMIP-1 and IL-8 cytokines. Although much more TNF-α mRNA accumulates after HA or SEB treatment than with anti-CD3 in the presence or absence of IL-2 (Fig. 5), the amount of TNF-α released into the culture medium is significantly higher in the cultures treated with anti-CD3 and IL-2 (~5800 pg/ml after 24 h) than in the T cells undergoing anergy induction (~3350 pg/ml 24 h after HA treatments and ~2600 pg/ml 24 h after SEB treatments) (Fig. 5). Finally, we have also noted increased levels of IL-4 mRNA after anergy-inducing treatments relative to anti- CD3 activation with or without IL-2 (Fig. 6).

DISCUSSION

This study shows that the tolerizable T cell clone HA1.7 expresses high levels of mRNA for several cytokines during the induction of the nonresponsive state of clonal anergy. In fact, tolerizing signals (treatment with either HA peptide 307–319 or the superantigen SEB) appear to be stronger and longer lasting inducers of mRNA for most of the cytokines examined than is cellular activation with anti-CD3 in the presence or absence of IL-2. Interestingly, however, although more mRNA accumulates after tolerizing treatments than after anti-CD3 and IL-2 activation, the amount of secreted cytokines is typically less after tolerance induction signals than after activation treatments. This suggests that specific posttranscriptional mechanisms of regulating the secretion of T cell cytokines may be in place during anergy induction. This appears to be the case not only for HA1.7, but for another tolerizable T cell clone, DE9, which, for the cytokines examined in this study, exhibits similar patterns of cytokine mRNA expression and protein production during anergy induction and cellular activation (our unpublished observations).

The data obtained raise several issues. Our findings provide definitive evidence that at least some T cells not only transcribe but also secrete IL-8. In contrast, Smyth et al. (12) have recently reported that purified T cells from PBL transcribe but do not secrete IL-8. The data in that report, coupled with the data presented suggests that T cells have the capacity to regulate IL-8 production posttranscriptionally and that some differentiation event may be necessary to enable T cells to actively secrete this cytokine.

Of more particular relevance to T cell anergy, the data

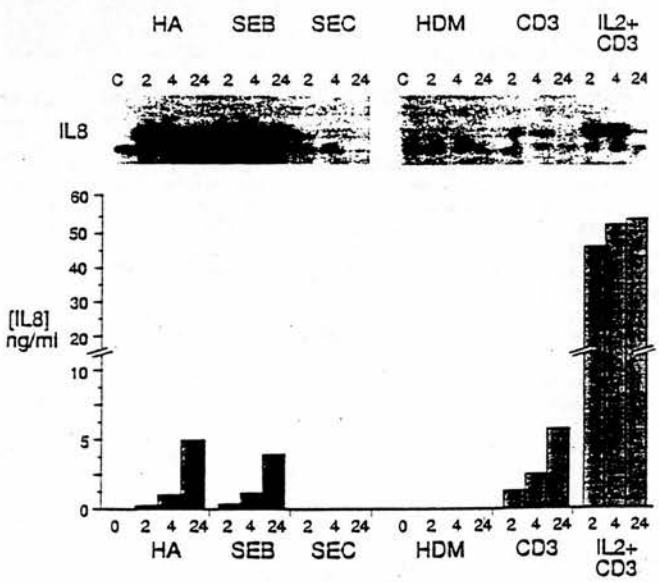


Figure 4. IL-8 mRNA expression and protein production by HA1.7 cells during anergy induction and activation. *Upper panel* depicts Northern blot analysis of IL-8 mRNA expression by HA1.7 cells in response to activation and tolerizing treatments listed at the top of the panel and as described for Figure 3. *Lower panel* represents ELISA analysis of secretion of IL-8 protein into the medium of HA1.7 cultures during the same treatments as above. IL-8 concentration is expressed as ng/ml.

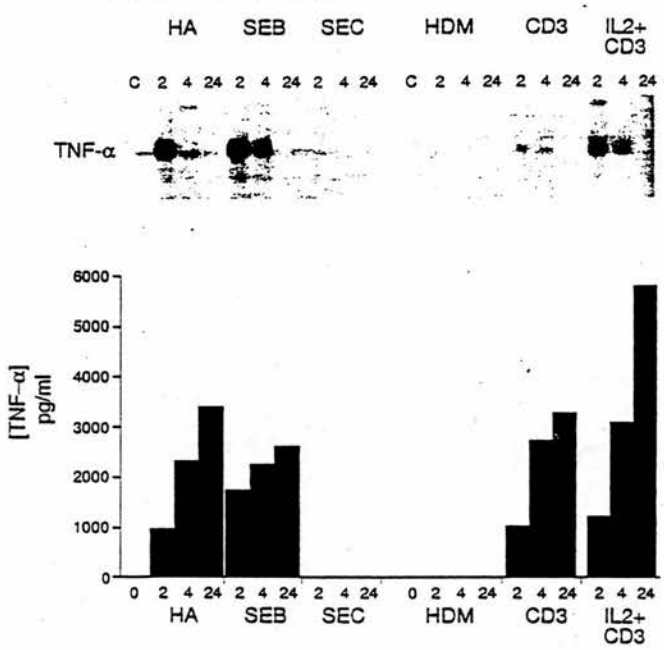


Figure 5. Analysis of TNF-α mRNA and protein secretion by HA1.7. Conditions are as listed for Figure 4. TNF concentrations as measured by ELISA are expressed as pg/ml.

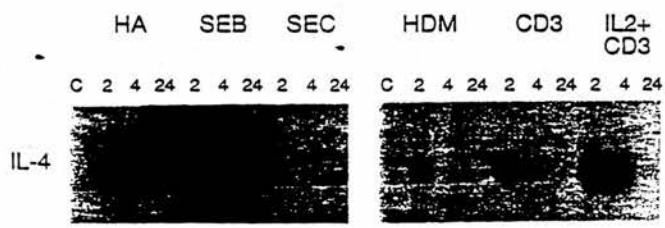


Figure 6. Northern blot analysis of IL-4 mRNA expression in HA1.7.

demonstrate that complete inhibition of T cell cytokine mRNA expression is not a general feature of T cell anergy induction, but there may exist specific mechanisms by which T cells undergoing anergy induction regulate cytokine secretion. These mechanisms are at present largely unclear, but the data are likely indicative of post-transcriptional control of cytokine production during the induction of anergy. Two possibilities are the formation of a "translational block" inhibiting cytokine protein synthesis in T cells undergoing tolerance induction, and increased degradation of cytokines in cells after tolerance induction relative to cells activated with anti-CD3 and IL-2. Inasmuch as the induction of tolerance appears to require new protein synthesis (38), the existence of specific "anergens" could be postulated to provide for such mechanisms.

It is possible that some of the cytokines examined may have roles in T cell regulation, and that the altering of their normal transcription and secretion could play a role in the process of anergy induction. It is likely that T cells express specific receptors for at least some of the cytokines examined in this study, based on chemotaxis data (26, 29), so autocrine or paracrine mechanisms of regulation might be envisaged to play a role during the induction of T cell anergy. The murine homolog of HuMIP-1α, MIP-1α (20), has been shown by Graham et al. (25) to be an inhibitor of hematopoietic stem cell proliferation. Because HuMIP-1α mRNA is superinduced during anergy treatments, and because HA-treated cells secrete the highest levels of HuMIP-1 proteins, it seems possible that HuMIP-1α could have an antiproliferative effect on HA1.7 during the induction of anergy or could, perhaps, inhibit T cells in the same microenvironment. However, HA1.7 cells anergized with SEB, although superinduced with respect to the amount of HuMIP-1 mRNA, secrete no more HuMIP-1 proteins than do HA1.7 cells activated with anti-CD3 treatment in the presence or absence of IL-2. This suggests that different interactions of HA and SEB ligands with the TCR, whereas both inducing anergy in HA1.7, also result in differential regulation of the secretion of the HuMIP-1 proteins. Differential production of IL-2 in response to HA and SEB stimulation has also been observed in HA1.7 (our unpublished observations).

The data in this study suggest that the induction phase of T cell anergy, the first 24 h after treatment with superantigen or supraimmunogenic doses of specific Ag,

is accompanied by changes in the profiles of cytokine transcription and secretion as compared to the same period after activation of these T cells with anti-CD3 and IL-2. Investigation of the molecular events underlying these changes, as well as further experimentation with recombinant cytokines such as IL-8 and the RANTES/SIS molecules may provide insight into the workings of immune tolerance.

Acknowledgments. We thank Greg Bennett and David Thompson at Genentech Immunology Research and Assay Technology for assistance with the IL-8 ELISA, and Baron Reed for the TNF-α ELISA. We are grateful to Dr. E. Amento for reviewing the manuscript, Evelyn Berry for typing, and to John Mak for technical assistance.

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Interleukin-2 can prevent and reverse antigen-induced unresponsiveness in cloned human T lymphocytes

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Accepted for publication 21 March 1988

SUMMARY

The exposure of human T-cell clones to supra-immunogenic concentrations of peptide antigen in the absence of accessory cells induces antigen-specific unresponsiveness. Using this model we have investigated the ability of cytokines to modulate the induction of, or reversal of, T-cell tolerance. Our findings demonstrate that interleukin-2 (IL-2), but not interferon-gamma (IFN-γ) or interleukin-1 (IL-1), is able to inhibit the induction of T-cell unresponsiveness in a dose-dependent fashion. Moreover, IL-2 was able to reverse established antigen-dependent T-cell unresponsiveness. In order to determine if modulation of IL-2 receptors is able to induce or abrogate unresponsiveness, the T-cells were treated with anti-Tac antibody alone or together with tolerizing concentrations of antigen. Anti-Tac antibody was neither able to induce nor inhibit the induction of tolerance. The application of this model in the manipulation of immune responses is discussed here.

INTRODUCTION

The presentation of antigenic determinants in an immunogenic form to T-helper cells is critical in the generation of immune responses (Lamb *et al.*, 1983). Although there are many reports describing components necessary for the successful activation of T-helper cells (Schwartz, 1985), far less is known about the events regulating and preventing responses to potentially immunogenic antigen. For this reason we have been interested in investigating the mechanism by which antigen may abrogate antigen-induced responses, a process that has been termed 'immunological tolerance'. Useful model systems have abounded for studying immunological tolerance in B cells (reviewed by Nossal, 1984) but not for T cells, until it was possible to generate T-cell clones that recognize specific peptides.

With human T-cell clones recognizing peptides sequences derived from influenza virus haemagglutinin (HA), it was observed that the incubation of the clones with the appropriate peptide antigen in the absence of accessory cells inhibited proliferation to subsequent stimulation with an immunogenic concentration of peptide and accessory cells (Lamb *et al.*, 1983).

The underlying mechanism of this phenomenon was associated with changes in the expression of differentiation antigens on the T-cell membrane (Zanders *et al.*, 1983). Concomitant with the down-regulation of the antigen recognition-receptor complex, 'CD3/Ti', is the enhanced expression of the CD2 and IL-2 receptor proteins (reviewed by Feldmann, Zanders & Lamb, 1985). However, the changes in membrane CD3/Ti following tolerizing concentrations of peptide are not reflected by an alteration in their mRNA levels: whereas, in contrast, transcripts of CD2 and IL-2 receptor are enhanced (Lamb *et al.*, 1987). Since the tolerized T cells expressed IL-2 receptors and proliferated in response to exogenous IL-2, lack of endogenous IL-2 production, as suggested by the failure to detect IL-2 mRNA, may account for the unresponsiveness *in vitro*. A key role for IL-2 in tolerance has also been identified in studies with neonatally tolerized mice where IL-2 breaks tolerance, inducing graft rejection (Maikovsky *et al.*, 1985).

In this study we have investigated the potential of cytokines IL-1, IL-2 and IFN-γ to inhibit the induction and to reverse antigen-dependent T-cell unresponsiveness. The results reported here indicate that IL-2 but not IL-1 or IFN-γ prevents the induction of tolerance and also reverses it once induced. Antibodies to the IL-2 receptor are unable to influence this process.

Abbreviations: APC, accessory cells; IFN-γ, interferon-gamma; IL-1, interleukin-1; IL-2, interleukin-2; PBM, peripheral blood mononuclear cells.

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MATERIALS AND METHODS

Antigen

Synthetic peptides of HA1: p14 (residues 306–320) was obtained from Peninsula Laboratories (Belmont, CA); p17 (residues 174–196) and p20 (306–329) were the kind gift of Dr R. A. Lerner, Scripps Research Institute, La Jolla, CA. The peptide p112 (residues 112–132) was from the 65,000 MW protein of mycobacteria and was the generous gift of Dr J. B. Rothbard, Imperial Cancer Research Fund, London.

Antibodies

Murine monoclonal antibodies, anti-IL-2 receptor (Tac), anti-CD3 (UCHT1) and anti-HLA class I (2A-1) were the generous gifts of Drs T. A. Waldmann, NCI, Bethesda, P. Beverley, ICRF, London, and M. Crumpton, ICRF, London, respectively.

Recombinant cytokines

Purified recombinant human IL-2 was obtained from Sandoz, Vienna, Austria, by courtesy of Dr E. Liehl. It had an activity of $\sim 10^6$ U/mg. Purified recombinant (IFN-γ) was supplied by Dr G. Adolf of Boehringer-Ingelheim, Austria. It had an activity of 2×10^7 U/mg. Purified recombinant human IL-1α and β were supplied by Dr P. Lomedico (Roche, NJ). IL-1α had an activity of 2×10^7 U/mg assayed in the thymocyte co-mitogenesis assay. IL-1β had an activity of 2×10^7 U/mg.

Isolation of antigen-specific T-lymphocyte clones

Influenza virus haemogglutinin (HA) reactive T-cell helper clone (HA 1·7, CD4$^+$) was isolated as described previously (Lamb *et al.*, 1982, 1983). Briefly, T cells from a 6-day culture of peripheral blood mononuclear leucocytes (PBM; $1{\cdot}5 \times 10^5$/ml) stimulated with immunopurified HA (0·1 μg/ml) in RPMI-1640 containing 10% human A$^+$ serum (Edgeware Blood Transfusion Centre, London) were cloned by limiting dilution (0·3 cells/well) in microtest II trays (Falcon, Oxnard, CA) with irradiated autologous PBM (5×10^5/ml), HA (0·1 μg/ml) and IL-2. Clones were expanded with fresh IL-2 every 3–4 days and irradiated PBM and influenza virus every 7 days. Prior to use in proliferation assays, the cloned T cells were rested 6–8 days after the addition of accessory cells.

Induction of unresponsiveness

In order to induce unresponsiveness, cloned T cells (5×10^4/ml) were cultured in 96-well round-bottomed plates for 16 hr with peptides or monoclonal antibody at different concentrations as indicated, alone or together, and then washed extensively before adding to the proliferation assays (Lamb *et al.*, 1982). In some cultures cytokines were added together with, or 30 min prior to, the tolerizing antigen.

In order to elute IL-2 from its receptor and so prevent carry over of IL-2, the T cells were incubated with 0·2 M glycine buffer (pH 3·8) for 10 min on ice (Fujii *et al.*, 1986), and then washed five times in RPMI-1640 supplemented with 10% human A$^+$ serum before addition to proliferation assays.

Proliferation assays

Cloned T cells (5×10^4/ml), after pretreatment in tolerance-induction cultures, were incubated with p14 (1·0 μg/ml) together with irradiated histocompatible PBM ($1{\cdot}25 \times 10^5$/ml) or with recombinant IL-2 in 96-well round-bottomed microtitre plates. In cultures designed to investigate the reversal of tolerance, IL-2 (1 μg/ml) was added to the cells after washing. At 60 hr of incubation, tritiated methyl thymidine ([^{3}H]TdR, 1 μCi/well; Amersham International, Amersham, Bucks), was added and the cultures harvested onto glass-fibre filters 8–16 hr later. Proliferation was correlated with [^{3}H]TdR incorporation, as measured by liquid scintillation spectroscopy. The results are expressed as mean counts per minute (c.p.m.) $\pm$ SEM for triplicate cultures.

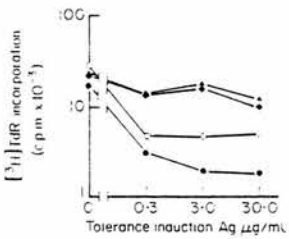

Figure 1. Ability of IL-2 to inhibit the induction of T-cell unresponsiveness. Clone HA1·7, 1×10^4, was tolerized $\pm$ varying concentrations of antigen (Ag), p14, in the presence of IL-2: ● no IL-2; ○ 0·1 μg/ml IL-2; + 0·3 μg/ml IL-2; ▲ 1·0 μg/ml IL-2, for 16 hr. Cells were washed extensively with acidic buffer to elute IL-2 from its receptor, then challenged with $2{\cdot}5 \times 10^4$ accessory cells plus the activating dose of p14 (1·0 μg/ml) for 3 days.

RESULTS

Ability of recombinant IL-2 to prevent the induction of T-cell unresponsiveness

Exposure to increasing concentrations (0·3–30 μg/ml) of p14 rendered T cells of clone HA1·7 unresponsive to an immunogenic challenge of p14 in the presence of accessory cells (Fig. 1). However, the addition of rIL-2 to the tolerance-induction culture inhibited the development of antigen-dependent unresponsiveness. The inhibition was dose-dependent since T cells incubated with 0·1 μg/ml were only partially resistant to the tolerizing effects of peptide, whereas 0·3 and 1 μg/ml of rIL-2 had marked effects (Fig. 1). It seemed unlikely that the proliferation observed was merely IL-2 carry over, since T cells pretreated with IL-2 were washed with an acidic buffer, pH 3·8, to elute IL-2 from its receptor (Fujii *et al.*, 1986). T cells pre-incubated with IL-2 alone and washed in this way failed to proliferate alone in culture or in the presence of accessory cells (Table 1, line 5, 727 c.p.m.). The specificity of this unresponsiveness was confirmed (Table 1), as p14 was effective but an unrelated peptide (p112) from the 65,000 MW protein of mycobacteria was not.

IL-1 and IFN-γ fail to inhibit the development of T-cell unresponsiveness

IL-1 is a growth factor involved in the activation of T cells. Therefore, it was of interest to determine whether or not IL-1 has the capacity to abrogate T-cell unresponsiveness. The addition of recombinant IL-1α at concentrations ranging from 1 to 1000 U/ml had no appreciable effect in the induction of tolerance with supra-immunogenic concentrations of p14 (Fig. 2, Table 2). Although incubation with IL-1α at 1000 U/ml overnight in the absence of peptide reduced the capacity of the T cells to respond to an immunogenic challenge with p14, this was not observed when peptide (10 or 100 μg/ml) was added to the cultures (Fig. 2). In addition, IL-1β had no inhibitory effect on antigen-induced unresponsiveness (data not shown).

The effects of IFN-γ on antigen-induced T-cell tolerance were similar to those of IL-1. While exposure to IFN-γ alone appeared to partially inhibit the response when challenged with p14, however with p14 present in the induction culture at 10 and

Table 1. IL-2 can inhibit the induction of T-cell unresponsiveness

Tolerance induction			Response (c.p.m.)		
T cells	Antigen	IL-2	APC	APC − p14	IL-2
HA1·7	−	−	192	14,131	7206
−	p14 (30 μg/ml)	—	283	1624	9431
−	p112 (30 μg/ml)	—	311	10,768	8826
−	p14 (30 μg/ml)	1 μg/ml	820	8713	11,473
−	−	+	727	17,215	12,762
−	−	−	241	326	—

Cloned T cells of HA1·7 (5×10^4/ml) were precultured in medium, IL-2, antigen (30 μg/ml) or the combination of antigen and IL-2 overnight and then washed prior to the addition to proliferation assays. In the proliferation assays, T cells (5×10^4/ml) were added to histocompatible irradiated PBM ($1{\cdot}25 \times 10^5$/ml) in the presence or absence of p14 (1 μg/ml) or cultured in IL-2 alone. Proliferation as correlated with [^{3}H]TdR incorporation was determined at 72 hr. Results are expressed as mean counts per minute (c.p.m.) for triplicate cultures. SEM were less than 15%.

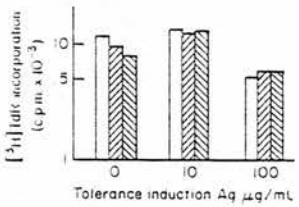

Figure 2. Failure of IL-1 to inhibit the induction of T-cell responsiveness. Clone HA1·7, 1×10^4, was tolerized ± varying concentrations of Ag p14, in the presence of recombinant IL-1α: □ no IL-1; ▨ 1 U/ml; ■ 1000 U/ml, for 16 hr. Cells were washed, then challenged with $2{\cdot}5 \times 10^4$ accessory cells plus activating dose of p14 (1 μg/ml) for 3 days.

Table 2. Failure of IL-1 and IFN-γ to inhibit the induction of T-cell unresponsiveness

	Response (c.p.m.)				
		IL-1		IFN-γ	
Tolerance induction antigen (μg/ml)	0	1 (U/ml)	1000 (U/ml)	100 (U/ml)	1000 (U/ml)
0	11,267	9028	8499	7362	8587
10	5931	6537	4965	6257	4039

Tolerance induction and proliferative assays were as described in legends to Figs 2 and 3.

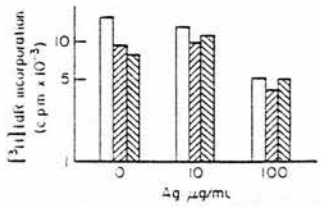

Figure 3. Failure of IFNγ to inhibit the induction of T cell unresponsiveness. Clone HA1·7, 1×10^4 was tolerized ± varying concentrations of Ag p14, in the presence of IFN-γ: □ no IFN-γ; ▨ 100 U/ml; ■ 1000 U/ml for 16 hr. Cells were washed, then challenged with $2{\cdot}5 \times 10^4$ accessory cells plus activating dose of p14 (1 μg/ml) for 3 days.

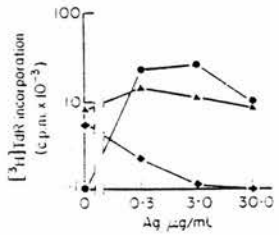

Figure 4. Ability of IL-2 to reverse antigen-specific unresponsiveness. Clone HA1·7, 1×10^4, was tolerized ± varying concentrations of Ag p14. Tolerance induction is indicated by ♦. Cells were washed, then challenged with 1·0 μg/ml IL-2, indicated by ▲. The normal control activation curve of 1×10^4 HA1·7 + $2{\cdot}5 \times 10^4$ accessory cells + varying doses of Ag, p14, is indicate by ●.

Table 3. Anti-IL-2 receptor (Tac) antibody neither inhibits the induction of nor induces unresponsiveness

Pretreatment		Response	
Antibody	Ag	APC − p20	IL-2
(a)			
—	—	8509 ± 903	7782 ± 614
—	p20	355 ± 89	8345 ± 791
—	p17	7224 ± 458	6815 ± 846
Anti-Tac	p20	803 ± 110	7160 ± 511
Anti-HLA class I (2A-1)	p20	824 ± 165	7717 ± 716
(b)			
—	—	10,282 ± 1142	4814 ± 438
—	p20	906 ± 242	6357 ± 511
—	—	8721 ± 631	3871 ± 142
Anti-Tac	—	9491 ± 783	4067 ± 337
Anti-HLA class I (2A-1)	—	10,461 ± 1391	4952 ± 920

Cloned T cells of HA1.7 were pretreated with antibody (5 μg/ml), antigen (30 μg/ml) or both together and after washing assayed as described in legend to Table 1.

100 μg/ml no difference was observed between the control cultures without antigen (Fig. 3, Table 2). These preparations of recombinant IL-1 and IFN-γ were effective in the thymocyte assay (IL-1) and monocyte HLA-DR induction (IFN-γ) in our laboratory (data not shown).

Reversal of antigen-induced unresponsiveness by IL-2

The presence of IL-2 during the initial incubation of peptide abrogates the induction of T-cell unresponsiveness (Fig. 1). Therefore, it was of interest to determine if IL-2 could reverse tolerance once established. Thus experiments were performed culturing T cells with IL-2 after the induction of unresponsiveness. Indeed, adding IL-2 to cultures containing T cells tolerized over a range of antigen concentrations, it was able to restore to ability to respond to p14 (Fig. 4). As the T cells were treated with acidic buffer to dissociate IL-2 from its receptor, it would appear that IL-2 is able to reverse the antigen-dependent unresponsiveness and that IL-2 carry over does not account for the proliferation observed. The apparent high concentration of IL-2 required to reverse tolerance 1 μg/ml (1000 U/ml) is due to its low biological activity.

Effect of anti-IL-2 receptor antibody (Tac) on the induction and reversal of antigen-specific unresponsiveness

Although anti-Tac antibody interacts with the binding site for IL-2, blocking both high and low affinity binding of IL-2, it does not stimulate IL-2 receptors or otherwise mimic IL-2, (Miyawaki *et al.*, 1982). Anti-Tac was used to determine whether or not the modulation of IL-2 receptors could interfere with the induction of antigen-dependent tolerance or induce unresponsiveness itself. The anti-Tac antibody, similar to the control anti-HLA class I antibody, was unable to inhibit p20-induced unresponsiveness (Table 3). Furthermore, pretreatment of the T cells with anti-Tac antibody alone did not induce unresponsiveness. As a specificity control for p20 induced tolerance, T cells were exposed to the unrelated HA peptide p17 and no unresponsiveness resulted (Table 3).

DISCUSSION

Although some of the mechanisms of T-cell activation in the induction of an immune response are becoming understood, there is little information on those regulating unresponsiveness. In human systems there could be significant clinical benefit in autoimmune or infectious diseases from understanding mechanisms of unresponsiveness in more detail (Feldmann, 1987b; Nath, 1983).

Neither is self non-responsiveness well understood. It is considered to be acquired in large part, during fetal development in the thymus. Recent studies of T-cell receptor Vβ gene expression in murine thymus have suggested that during T-cell maturation there may be deletion of clones using certain Vβ genes, as judged by their presence in the immature ($CD4^+$ $CD8^+$) cells but absence from the mature ($CD4^+$ or $CD8^+$) T cells (Kappler, Roehm & Marrack, 1987). A role for suppressor cells in self non-responsiveness has been asserted on the basis of a variety of studies, but the relative inability to clone suppressor T cells (Lamb & Feldmann, 1982) makes it difficult to evaluate their role in self non-responsiveness. The process of antigen-induced non-responsiveness of T-cell clones has been reported by ourselves using human class II-expressing T cells (Feldmann *et al.*, 1985), and more recently a murine model using class II-negative T cells and fixed class II-expressing APC has been developed (Jenkins & Schwartz, 1987). Although both the human and murine systems appear analogous, there is insufficient data to relate these *in vitro* processes to the mechanisms of 'immunological tolerance' as they occur during fetal development and subsequently *in vivo*, although the MHC restriction both *in vivo* and *in vitro* is suggestive of similarities (Matzinger, Zamoyska & Waldmann, 1984; Lamb & Feldmann, 1984).

The addition of a tolerizing concentration of antigen down-regulates the CD3/Ti-receptor complex concomitant with enhanced expression of IL-2 receptor (Zanders *et al.*, 1983; Feldmann *et al.*, 1985; Lamb *et al.*, 1987). Therefore, we tested whether or not engaging the IL-2 receptor with IL-2 or anti-IL-2 receptor antibodies was able to block the induction of, or reverse, tolerance. The results presented here indicate that IL-2, but not IL-1 or IFN-γ, at concentrations effective in bio-assays, can abrogate the induction of unresponsiveness at concentrations effective in bio-assays. The difference between the effect of IL-2 and the ineffectiveness of anti-Tac may be due to the much greater affinity of the former (kD $\sim 10^{-12}$ mole/litre) compared to the latter (kD $\sim 10^{-9}$ mole/litre), or to the need for IL-2 to be internalized (reviewed by Leonard *et al.*, 1982). IL-2 can also reverse the unresponsiveness, if added after the tolerance-induction phase. Although murine T-cell clones tolerized *in vitro* are unable to secrete IL-2, the addition of IL-2 during the induction phase fails to prevent unresponsiveness developing (Jenkins *et al.*, 1987). However, it has been reported that IL-2 injected into neonatally tolerant mice can induce graft rejection (Malkovsky *et al.*, 1985).

From our findings, based on the control experiments and dose responses, it is possible that IL-2 acts at both stages. Particularly telling is the fact that reversal requires high concentrations of IL-2, being optimal at 1 μg/ml (1000 U/ml), whereas blocking of the induction occurs at lower levels (300 U/ml). In comparison, maximum proliferation of the cells was induced with approximately 30 U/ml. Thus it seems unlikely that the breaking is due to IL-2 carry over, as the cells were washed, reducing considerably the IL-2 carry over to the 'breaking or reversal' time.

The effect of IL-2 on antigen-induced unresponsiveness has certain implications for other systems. An obvious example is the induction of human autoimmunity. It is conceivable that activation of T cells by viruses or parasites may provide sufficient IL-2 in a local site to overcome unresponsive status and generate helper cells to certain autoantigens. The relevance of this to the classical autoimmune diseases is not known, however, we have found that there is abundant IL-2 mRNA in tissue biopsies of active autoimmune sites such as rheumatoid arthritis (Buchan *et al.*, 1988). There is the development of a number of autoantibodies in individuals parasitized with schistosomes and conceivably the pathway described above may be of relevance (reviewed by Feldmann, 1987a).

Recently, IL-2 has been used in tumour immunotherapy. By culturing blood mononuclear cells with 1000 U/ml of IL-2, activated killer cells are generated. These are known as lymphokine-activated killer cells or LAK cells (Muul & Rosenberg, 1986). Therapy with such cells causes considerable side-effects.

It is possible that some of the side-effects may be due to abrogation of self-tolerance in some T cells.

Our interest in analysing the effects of lymphokines and cytokines on the induction of T-cell unresponsiveness was primarily because of the possible insights that may be shed on the mechanisms, and consequently their potential for immune manipulation. Taken together, our results suggest that supra-immunogenic concentrations of antigen in the absence of accessory cells may prevent the production of IL-2, perhaps by switching off the IL-2 gene. It was proposed that the molecular basis for this may be an increase in intracellular calcium (Jenkins *et al.*, 1987). From *in vitro* studies with murine T-cell clones it appears that IL-2 is the only lymphokine that is switched off during tolerance induction (Jenkins *et al.*, 1987), and it remains to be determined whether human T cells are regulated in the same way.

ACKNOWLEDGMENTS

We thank Drs P. Beverley, M. Crumpton and T. Waldmann for gifts of monoclonal antibodies, Drs E. Liehl (Sandoz) and M. Malkovsky for recombinant IL-2, Dr G. Adolf (Boehringer-Ingelheim) for recombinant IFN-γ, Dr P. Lomedico (Roche) for recombinant IL-1α and β, Drs R. Lerner and J. Rothbard for synthetic peptides; the MRC and ICI plc for financial support; and Mandy Wilcox for typing this manuscript.

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International Immunology, Vol. 5, No. 5, pp. 461–466

CD28 mRNA rapidly decays when activated T cells are functionally anergized with specific peptide

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Key words: T cell tolerance, mRNA regulation, CD28

Abstract

The induction of non-responsiveness in specific clones of T cells *in vivo* might be expected to reverse immunologically mediated disease processes. With this goal in mind, experiments *in vitro* with cloned T cells have investigated the mechanisms of induction of anergy. Resting T cells can be functionally inactivated *in vitro* by high doses of appropriate peptide in either the presence or absence of antigen presenting cells. During the induction of anergy, the modulation of the surface phenotype of T cells is similar to that of cells proliferating in response to an immunogenic stimulus. The amount of T cell receptor at the cell surface is down regulated, whereas the CD2 and CD25 receptors are increased in density. In contrast, CD28 has been identified as a membrane protein that is differentially regulated during activation and the induction of non-responsiveness. Ligation of CD28 provides an efficient costimulatory signal for activation of T cells. In this report, we show that not only resting cells, but also fully activated T cells can be rendered non-responsive and that this process is accompanied by profound downregulation of CD28, both at the level of cytoplasmic mRNA and surface expression of the mature protein. This observation anticipates clinical intervention in immunologically mediated disease, where the target T cells are more likely to be activated than in a resting state.

Introduction

The phenomenon of immunological tolerance is well described; it results from both deletions of sets of T lymphocytes in the thymus and from the maintenance of some sets in a state of anergy in the periphery. The mechanisms of post-thymic T cell tolerance have been studied *in vitro* and several model systems have now been well characterized (reviewed in 1). For human CD4$^+$ T cells it has been established that stimulation with high doses of appropriate peptide, in either the presence or absence of antigen presenting cells (APC), will inhibit subsequent antigen dependent proliferation (2). The phenotype of T cells during the induction phase of anergy parallels that of cells which received an activating signal. Transcription of several genes is hyper-induced during this period; however, the ability of anergized T cells when rechallenged to release a range of cytokines is curtailed (3). Anergic T cells fail to produce enough IL-2 by the normal autocrine pathway to deliver a growth signal that permits clonal expansion and so anergy may be considered as abortive activation. However, the cells remain viable and they will proliferate, in response to exogenous IL-2 (4). In humans, both T_h1 and T_h2 subtypes of CD4$^+$ T cells are susceptible to peptide-mediated anergy. Interestingly, anergized T_h2 type T cell clones do not produce IL-4, but release interferon (IFN)-γ when rechallenged (5). This observation suggests that a strategy of peptide vaccination for desensitization of allergic individuals may divert the overall immune response towards the T_h1 type and thus away from IgE synthesis. In contrast, this study also revealed that T cell clones of the T_h1 type did not release IFN-γ when rechallenged. Murine CD4$^+$ T cell clones cannot be anergized by high doses of peptide, probably because these T cells do not express MHC class II molecules. However, these cells do not proliferate when stimulated with antigen and chemically modified APC and they remain refractory to a subsequent immunogenic rechallenge (6). Unlike the human system, murine T cell anergy is immediate and not characterized by a change in surface phenotype.

After an activating signal, T cells upregulate their expression of the CD28 antigen. In contrast, T cells that receive a tolerizing signal downregulate their expression of the antigen (7). This reduction of CD28 has been reproducibly seen in human T cells representative of the T_h1- and T_h2-like subsets. A signal through

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Transmitting editor: M. Neuberger

Received 9 November 1992, *accepted* 22 January 1993

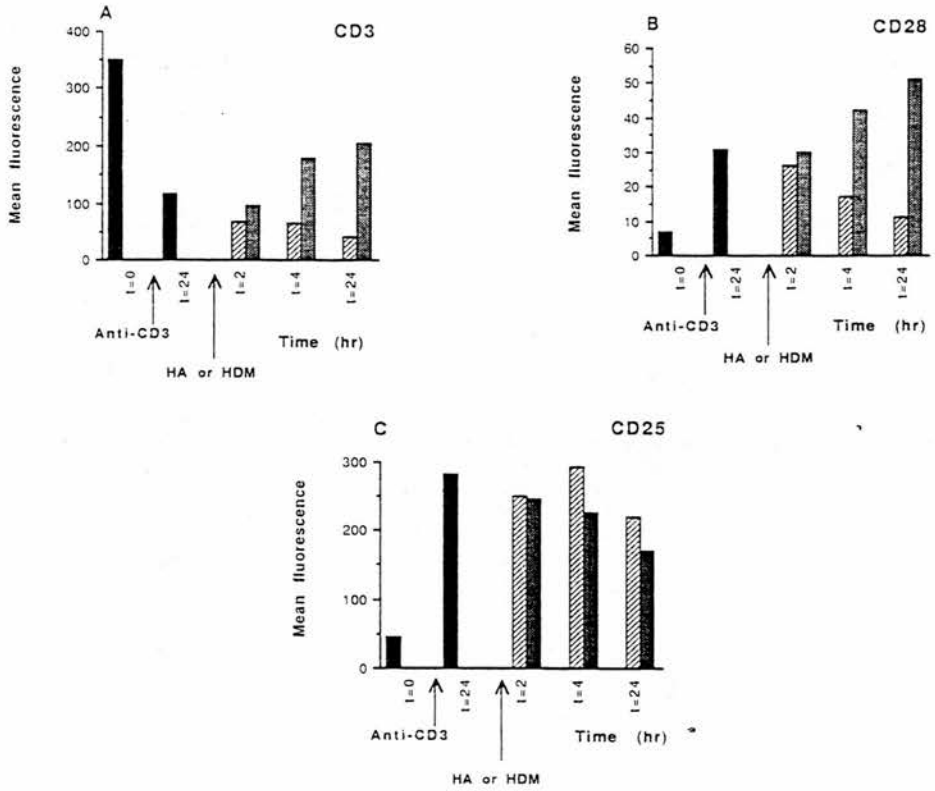

Fig. 1. Phenotypic modulation of activated T cells exposed to anergizing concentration of specific peptide. Membrane expression of CD3 (A), CD28 (B), and CD25 (C) of activated T cells exposed to high concentrations of HA 307 – 319 (cross-hatched bars) or HDM 1 – 15 (stippled bars) were compared by flow cytometry at 2, 4 and 24 h after activation. Expression of CD3, CD28, and CD25 for resting T cells and those activated by anti-CD3 antibody was also determined (solid bars in A, B, and C).

CD28 is a pleiotropic event: it is costimulatory for T cell proliferation (8) and results in the upregulation of several T cell derived cytokines by stabilizing their mRNAs (9,10). Ligation of CD28 affects IL-2 synthesis by the generation of a nuclear complex that increases transcription from the IL-2 gene promoter (11). It is now clear that failure to ligate CD28 is the tolerogenic event when T cells are stimulated by antigen and chemically modified APC (12), probably because the natural ligand of CD28, the activation antigen B7/BB-1 (present on dendritic cells, activated B cells, and monocytes), is denatured in the fixation process. It seems possible then, that tolerance induced by high dose peptide antigen is similar to tolerance induced by chemically modified APC. In the former, absence of a costimulatory signal can be ascribed to low receptor expression, in the latter to lack of ligand. High doses of peptide cause down regulation of CD28 so that no second signal can be delivered on rechallenge whereas fixation of APC causes the disruption of the natural ligand with the same consequence. The present report extends these observations. We show that downregulation of CD28 after a tolerogenic signal occurs rapidly and by post-transcriptional mechanisms. Furthermore, we establish that activated T cells, involved in an ongoing immune response, can be anergized by high concentrations of free peptide.

Methods

Peptides

The influenza haemagglutinin peptide (residues 307 – 319: HA 307 – 319) and control peptide from the group II allergen of the house dust mite (residues 1 – 15; HDM 1 – 15) were synthesized using standard solid-phase technology on an Applied Biosystems model 431A synthesizer, purified by reverse-phase HPLC. The constitution of the peptides was confirmed by amino acid analysis as previously described (13).

Antibodies

Flow cytometric analysis was performed using fluorescein-conjugated murine mAbs. Anti-Leu4 (CD3), anti-IL-2 receptor (CD25) and a mouse IgG1 control were bought from Becton-

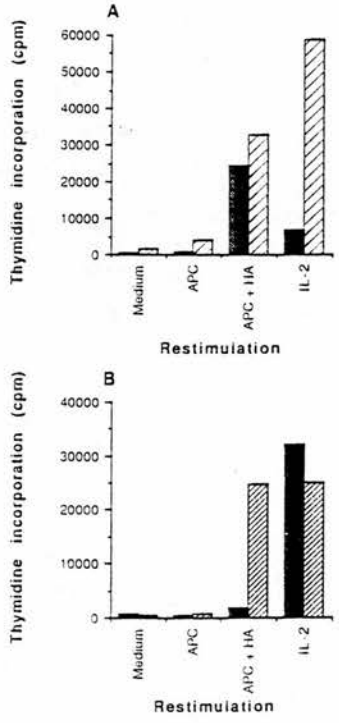

Fig. 2. Ability of specific peptide to induce anergy in activated T cells. In (A), cloned T cells of HA1.7 (10^6/ml) were pretreated by culturing in medium alone (stippled bars) or with insolubilized anti-CD3 antibody and IL-2 (cross-hatched bars) for 24 h. After pretreatment the T cells (10^5/ml) were assayed for their ability to respond to restimulation with HA 307-319 (0.3 μg/ml) and HLA-DR1$^+$ APC (irradiated PBMC) or IL-2 alone. Control responses of the pretreated T cells to APC alone or medium alone were measured. Proliferation as correlated with [^{3}H]TdR incorporation was determined at 72 h. In (B), T cells initially stimulated with anti-CD3 antibody and IL-2 for 24 h were exposed either to HA 307 – 319 (solid bars) or HDM 1 – 15 (cross-hatched bars), both at 50 μg/ml, for a further 24 h. The T cells were then assayed for their ability to respond to restimulation as described above.

Dickinson (Oxford, UK). Anti-CD28 antibody was bought from Janssen (Alde, Germany). The murine anti-CD3 antibody used for cell activation was a kind gift from H. Spits (DNAX, Palo Alto, CA).

HA reactive cloned T lymphocytes

The isolation of HA1.7, a clone of T cells reactive with HA 307 – 319, has been reported in detail previously (2,7). Briefly, peripheral blood mononuclear leukocytes (PBMC) were stimulated with purified HA in RPMI 1640 (Gibco, Uxbridge, UK) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 5% human AB$^+$ serum. The activated T cells were cloned by limiting dilution in the presence of irradiated autologous PBMC, IL-2, and antigen. Growing HA1.7 cells were expanded by stimulation with antigen and filler cells every 7 days. After stimulation, the cells were expanded with IL-2 every 3 – 4 days. The cells were rested 7 – 8 days after the last addition of filler cells and antigen before their use in experiments.

Experimental protocol

Resting cloned T cells (10^6/ml) were activated by incubation with anti-CD3 antibody insolubilized on tissue culture plates (12 μg/ml) together with 10% IL-2. Activated T cells were washed, then incubated with HA 307 – 319 (50 μg/ml). Control cultures were treated with the same concentration of an irrelevant peptide (HDM 1 – 15). After 24 h the cells were washed three times and then assessed for their ability (10^5/ml) to respond to an immunogenic challenge by APC [irradiated histocompatible (DR1$^+$) PBMC 1.25×10^5/ml] and antigen (HA 307 – 319: 0.3 μg/ml).

Proliferation assays

After a further 60 h incubation, tritiated methyl thymidine ([^{3}H]TdR: 1 μCi/well; Amersham International, Amersham, UK) was added and the cultures harvested 12 h later. Proliferation as correlated with [^{3}H]TdR incorporation was measured by liquid scintillation spectroscopy. The results are expressed as mean counts per minute (c.p.m.) for triplicate cultures. In all experiments the SEM was <20%.

Determination of phenotypic expression by fluorescence flow cytometry

T cells were stained directly using saturating concentrations of fluorescein-conjugated murine monoclonal antibodies. Propidium iodide exclusion was used to identify viable cells. Cell populations were analyzed by flow cytometry using an Epics-Profile II (Coulter, Luton, UK). Mean fluorescence intensity was measured on a linear scale. All viable cells were included in each determination and in each case the fluorescence profile was unimodal.

Oligonucleotide synthesis

Oligonucleotides were synthesized using an Applied Biosystems (Cheshire, UK) PCR-mate-EP 391 DNA synthesizer, and purified using oligonucleotide purification columns (Applied Biosystems), according to the manufacturers general instructions. The sequences used were:

CD28 forward 5′-CTT GTA GCG TAC GAC AAT GCG GT-3′
CD28 reverse 5′-AGG GCT GGT AAT GCT TGC GGG-3′
Actin forward 5′-GTG GGG CGC CCC AGG CAC CA-3′
Actin reverse 5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′

Analysis of specific mRNA levels by polymerase chain reaction (PCR)

Total cellular RNA was prepared by guanidinium thiocyanate – phenol/chloroform extraction (14). The RNA from each sample was then transcribed to cDNA using 20 U AMV reverse transcriptase (IBI, Cambridge, UK) and oligo(dT)$_{12-18}$ as a primer. Transcripts were amplified through 25 cycles of PCR using appropriate primers at a final concentration of 0.25 μM. Amplification reactions were carried out in a Perkin Elmer thermal cycler (Cetus; denaturation, 1 min, 94°C, annealing, 1 min, 55°C, extension, 2 min, 72°C). Reaction products were extracted with chloroform and 25 μl were blotted onto Hybond nylon membranes (Amersham) using a slot blot apparatus (Schliecher & Schuell, Dassel, Germany) and hybridized as previously described (15). Probes were selected from areas internal to the

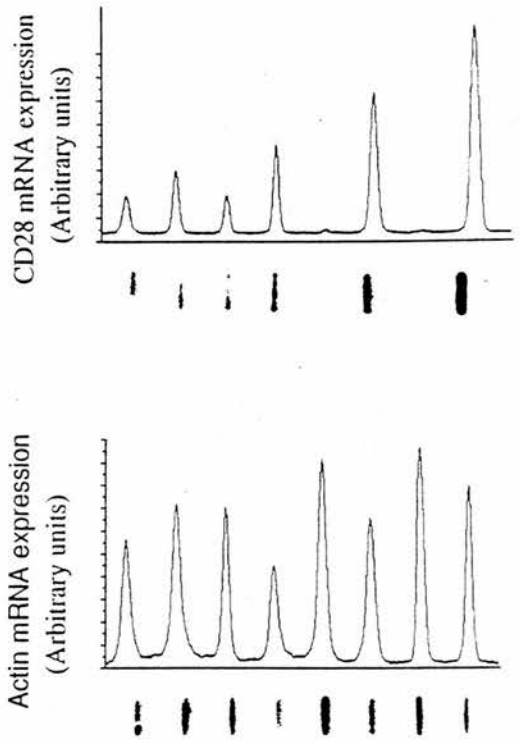

Fig. 3. Cytoplasmic mRNA levels in activated T cells exposed to anergizing concentration of specific peptide. Slot blotting and densitometric analysis of PCR products was used to measure the amount of mRNA encoding CD28 (upper panel) and cytoplasmic β-actin (lower panel). Resting T cells (lane 1) were pretreated by culturing with insolubilized anti-CD3 antibody and IL-2 for 24 h (lane 2). After pretreatment the T cells were exposed to either HA 307 – 319 (lanes 3, 5, and 7) or HDM 1 – 15 (lanes 4, 6, and 8), both at 50 μg/ml, and the T cells were harvested 2 h (lanes 3 and 4), 4 h (lanes 5 and 6), and 24 h (lanes 7 and 8) later.

two primers used in the amplification step and were labelled (specific activity $>10^8$ d.p.m./μg) using the random hexanucleotide priming method (16). The amount of mRNA in the original sample was then quantified by radiography and densitometry. Each sample was amplified with β-actin primers and processed in the same way to allow the starting levels of cDNA to be compared.

Nuclear run off analysis

Nascent RNA was labelled using [α-^{32}P]UTP (Amersham) as described (17) and hybridized to immobilized complementary sequences as detailed above. 1053/1, 1100/1, and 1057/1 are genomic clones covering the CD28 locus. These clones were a kind gift from K Lee (University of Michigan) [see (18) for details]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), CD2, and actin cDNA probes were a gift from M. Owen (ICRF, London, UK).

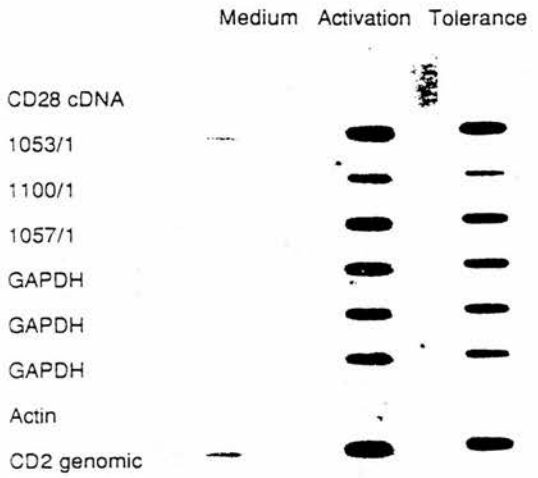

Fig. 4. Nuclear run off analysis, showing *de novo* transcription in isolated nuclei from cloned T cells, either untreated (Medium) or 24 h after induction of activation or tolerance. Nascent RNA was labelled and hybridized to immobilized complementary sequences: 1053/1, 1100/1 and 1057/1 are genomic clones covering the CD28 locus (see 18 for details).

Results

Phenotypic modulation of activated T cells after treatment with HA 307 – 319

The phenotypic modulation of resting HA 307 – 319 reactive CD4$^+$ T cells (HA 1.7) after treatment with activating and tolerizing regimens has been reported elsewhere (19). T cells were rested for 7 days after the last cycle of expansion, then activated with anti-CD3 antibody and IL-2. Twenty-four hours later, aliquots of cells were exposed to a tolerizing concentration of HA 307 – 319 or an irrelevant HDM peptide, and samples were analysed by fluorescence flow cytometry to determine the level of surface CD3, CD25, and CD28 after a further 2, 4, and 24 h (Fig. 1). Consistent with our previous findings, the cells had markedly downregulated membrane CD3 expression 24 h after an activating signal and, in the absence of any other signal CD3 expression slowly recovered over the following 24 h period. In contrast, CD3 expression on the cells that were treated with a tolerizing concentration of HA 307 – 319 was further reduced over the course of the experiment (Fig. 1A). IL-2 (CD25) expression was unremarkable: cells that received an activating signal showed an increase in receptor expression and this increase was not modulated by subsequent exposure to a tolerogenic signal (Fig. 1C). CD28 was upregulated after an activating signal and in the absence of any additional signal the expression of CD28 at the cell surface continued to increase (Fig. 1B). The activated cells treated with tolerogenic HA 307 – 319 downregulated CD28 from the surface, so that 24 h after a tolerizing signal the amount of CD28 was similar to the resting cell level. This rapid loss of CD28 from the cell surface indicates that the protein has a high turnover rate.

Induction of T cell anergy by treatment of activated cells with HA 307 – 319

The T cells were tested for their ability to respond to specific peptide, 24 h after receiving an activating signal delivered through the T cell receptor (Fig. 2A). The data show that pre-activation does not affect the ability of cells to proliferate in response to peptide presented in an immunogenic form. Indeed the activated cells are hyperresponsive to challenge. This phenomenon is due to the induction of CD25 and the manifest hyper-responsiveness of activated cells to IL-2 (Fig. 2A). These experiments were designed to test whether activated cells subsequently treated with a high dose of specific peptide could be functionally inactivated. After incubation with HA 307 – 319 (50 μg/ml) for 24 h, the T cells failed to proliferate when restimulated with HA 307 – 319 presented in an immunogenic form (Fig. 2B). In contrast, T cells incubated with HDM 1 – 15 responded to rechallenge with specific peptide appropriately presented. The addition of exogenous IL-2 caused proliferation of anergized cells, confirming functional inactivation was not the result of cytolysis. To our knowledge this is the first demonstration that activated human T cells can be rendered functionally non-responsive.

CD28 transcripts after activation and superinduction of T cell anergy

In order to examine the kinetics of CD28 mRNA production during the induction of non-responsiveness, levels were determined at 2, 4 and 24 h after exposure to the anergizing signal (Fig. 3). Quantitative PCR analysis was used to measure the level of CD28 mRNA in cells that were treated with a high dose of peptide antigen or irrelevant peptide as a control. CD28 mRNA accumulated in T cell clones after activation and continued to accumulate when the cells were treated with an irrelevant peptide. In contrast, cells treated with a tolerizing dose of HA 307 – 319 had markedly less CD28 message 2 h later, and 4 h after treatment CD28 mRNA was virtually undetectable. These data correlate well with expression of CD28 at the cell surface after the various treatments. Clearly, CD28 mRNA is rapidly degraded during the induction phase of tolerance. However, it is not clear from these experiments whether CD28 downregulation occurs at the level of transcription or because of increased specific catabolic activity.

Nuclear transcripts of CD28 after activation and induction of T cell anergy

Nuclear run off assays were performed to investigate transcriptional activity in cells that received either an activating signal or a tolerizing signal. The data suggest that increased expression of CD28 after activation is a consequence of transcriptional upregulation (Fig. 4). It is important to note that these data do not rule out attenuation as the primary mechanism of transcriptional control. On the contrary, it is noteworthy that λ clone 1100/1 (Fig. 4) contained DNA from the most 3′ end of the gene and is apparently unrepresented as a transcript in the nucleus of cells cultured in medium alone. This suggests that transcription is attenuated in unstimulated cells and that a positive signal permits full length transcription through the remainder of the gene. Our results also show that cells that received a tolerizing signal transcribe the CD28 gene at the same rate as cells undergoing activation. Different levels of CD28 mRNA accumulate in the two populations, probably because of differential rates of degradation after treatment. The biological importance of post-transcriptional mechanisms of gene regulation is now being generally recognized (20). Variations in the steady state level of cytoplasmic mRNA can be attributed to regulation at any point in the pathway between nuclear transcription and degradation in the cytoplasm, e.g. changes in RNA processing and transport. However, mRNA stability is the best candidate mechanism: it is a known target for regulatory mechanisms in T cells and indeed CD28 signalling affects mRNA stability itself.

Discussion

T cell activation is usually a prelude to proliferation, which is driven at least in part by growth factors, notably IL-2. During the activation of T cells, occupancy of the T cell receptor is conventionally referred to as signal 1 and a growth signal referred to as signal 2. Bretscher and Cohn (21) first proposed this nomenclature to explain self – non-self discrimination and their model demanded that signal 2 be antigen specific and able to distinguish self from non-self. Signal 2 is, therefore, necessarily a function of an immune effector population. The T cell growth factors fulfil this requirement so long as they are produced in an antigen dependent manner. A costimulatory signal for T cell activation is provided by ligation of C28 on the T cell to the accessory molecule B7/BB-1 on the surface of dendritic cells, B cells, and monocytes. It is likely, though it is not yet known whether other receptors can transduce an effective costimulatory signal. The net effect of appropriate stimulation of T cells is the synthesis and release of IL-2 and it has been suggested that signal 1 in the absence of costimulation leads to anergy because IL-2 is not released (22,23). However, it is clear from work with human T cell clones that an enhanced signal 1, delivered through the T cell receptor, even in the presence of antigen presenting cells and therefore a costimulatory signal, is sufficient to induce non-responsiveness (5). These experiments clearly showed that the optimum dose of peptide for the induction of anergy was increased in the presence of APC. It is possible that T cells are rendered susceptible to the induction of anergy after stimulation through the T cell receptor by the production of factors that have been termed anergens (3). These are likely to be a diverse group of molecules that may affect processes in distinct cellular compartments (see below). Rescue from anergy may occur by the transduction of an appropriate signal 2, which at its most simplistic might be a costimulatory signal that is sufficient to upregulate IL-2 production. Anergic T cells will proliferate in response to exogenous IL-2 (4,23) and it has been suggested that this growth dilutes anergens to a level low enough to allow a normal proliferative response on rechallenge (23). An enhanced signal 1 by stimulation of T cells with a high density of antigen might therefore be expected to result in a high level of anergens, which, beyond a certain level, a costimulatory signal cannot override. This suggests that costimulation must act negatively on the induction of anergens as well as positively on the production of signal 2.

Induction of anergy is an active process that requires protein synthesis (24,25). Anergens will include some of these molecules as well as activation or translocation of other effector molecules. For example, *de novo* protein synthesis probably requires transcription of the relevant genes and would therefore be

characterized by changes in the distribution of various transcription factors. One consequence of a tolerogenic signal, described in the present report, is the rapid decay of CD28 mRNA. It follows therefore, that one member of the family of anergens is a factor that can differentially control mRNA stability. Such a factor might be a sequence specific RNase or a factor that displaced a specific RNA protecting molecule. Future work will investigate these possibilities, ask whether the degradation accelerating factor is a newly synthesized protein, and examine the range of mRNA species affected by a tolerogenic signal. In addition, we will test other T cell clones to show whether the phenomenon is general or restricted to the T_h1-like phenotype. Interestingly, Takahana and Singer (26) have recently identified a post-transcriptional mechanism that regulates early thymocyte development. The mechanism is influenced by signals through the TCR and has similiar characteristics to the decay accelerating factor described in this paper. However accomplished, down modulation of CD28 from the cell surface could contribute to a mechanism whereby cells can be rendered anergic. Ligation of CD28 results in the upregulation of IL-2 by stabilizing its mRNA (9) and through the generation of a nuclear complex that increases transcription from the IL-2 gene promoter (11). Thus, the loss of CD28 from the surface of the T cell will render the costimulatory pathway ineffective. The rapid kinetics of CD28 down modulation is consistent with the short time (24 h) required for the induction of anergy.

The findings reported here demonstrate that immunogenic peptides, appropriately presented at high density, can inactivate specific clones of T cells that have been activated through the T cell receptor for antigen. The practical value of this observation is in the development of peptide-based approaches to immunotherapeutic intervention during the acute phase of autoimmune disease and specific allergic inflammatory responses.

Acknowledgements

We thank Ita Askonas for helpful discussions. This work was supported by the Medical Research Council (MRC) and Wellcome Trust. R.E.O'H. is a Wellcome Senior Clinical Research Fellow.

Abbreviations

AMV	avian myeloblastosis virus
APC	antigen presenting cell
dT	deoxythymidine
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HA	haemagglutinin
HDM	house dust mite
IFN-γ	interferon-γ
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
[^{3}H]TdR	tritiated methyl thymidine

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Biochemical events initiated by exposure of human T lymphocyte clones to immunogenic and tolerogenic concentrations of antigen*

Interleukin 2-dependent helper T cells, cloned from human peripheral blood lymphocytes activated with strain A influenza virus hemagglutinin, proliferate in response to a 24-residue synthetic peptide (p20) of hemagglutinin, but become unresponsive to a subsequent immunogenic challenge when pretreated with a high concentration of p20. This phenomenon is associated with a loss of the T3 antigen complex, presumably in association with the T cell receptor. We have examined this phenomenon in more detail and show that in addition to changes in the expression of T3 molecules on the cell surface, high doses of p20 cause changes in the expression of certain biosynthetically and surface-labeled proteins, although total DNA and protein synthesis was unaltered. Thus, by examining these biochemical phenomena we can begin to define some of the processes which occur during antigen activation of human T lymphocytes at the clonal level.

1 Introduction

Recent technical advances have made it possible to clone T lymphocytes and perform experiments which would have been uninterpretable using heterogeneous populations containing rare antigen-specific cells. We have used human T cell clones induced with the hemagglutinin-1 molecule of influenza which recognize a 24 amino acid peptide (p20, ref. [1]) to study the induction of antigen-specific, antigen-induced nonresponsiveness *in vitro*, a form of immunological tolerance. Optimal tolerance required 18 h exposure to high concentrations of antigen, and was more profoundly induced in the absence of antigen-presenting cells [2]. During the process of antigen-induced tolerance, the surface antigen T3 was selectively lost from the cell surface in proportion to the concentration of p20 used for incubation [3]. Functional tolerance (lack of response to antigen) was mimicked by pretreatment with anti-T3 which removed it from the cell surface. Since Meuer et al. have shown that "clonotypic" molecules corresponding to the T cell receptors for antigen are comodulated with T3 [4], our current concept is that during tolerance antigen at high doses down regulates the antigen receptor and takes the T3 complex with it.

Although this is a simple model for antigen-driven unresponsiveness at the clonal level, it is evident that other changes occur during exposure of HA1.7 to p20. We wished to characterize these changes by using standard biochemical techniques to study the fate of macromolecules during the period of preincubation with antigen and to evaluate this system as a model for antigen-T cell interactions. We show here that several changes in the expression of proteins occur, although the relevance of this to T cell activation phenomena in general is still unknown.

* This work was supported by the Imperial Cancer Research Fund, N. I. H. and the Arthritis and Rheumatism Council.

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Abbreviations: **IL 2:** Interleukin 2 **PBS:** Phosphate-buffered saline **PMSF:** Phenylmethylsulfonyl fluoride **SDS-PAGE:** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis **BSA:** Bovine serum albumin **NP40:** Nonidet-P40

2 Materials and methods

2.1 Cells, antigens and antisera

Clone HA1.7 was isolated and cultured as previously described [1]. For the experiments reported here, cells were used between 4 and 7 days after feeding with irradiated presenting cells and specific antigen. Peptides of the hemagglutinin-1 (HA-1) molecule of influenza virus hemagglutinin were synthesized according to the amino acid sequence of A/Hong Kong/X47 (H3N2) [5] as predicted from the nucleotide sequence [6]. p20 corresponded to residues 306–329, and p11 to 105–140. Both were kind gifts of Dr. R. A. Lerner, Research Institute of Scripps, La Jolla, CA.

2.2 Biosynthetic labeling of protein and DNA

For determining the effects of p20 concentration on DNA and protein synthesis, cells were washed once in HEPES-buffered RPMI 1640 (Gibco, Grand Island, NY) containing 10% A^- serum and cultured in 200 µl wells of microtest plates (Falcon Plastics, Oxnard, CA) in the same medium. Increasing concentrations of peptide dissolved in phosphate-buffered saline (PBS) were added, followed by radioisotope. (a) For DNA synthesis 2.5×10^4 cells were cultured overnight with 1 µCi = 37 kBq [^{3}H]thymidine (Radiochemical Centre, Amersham, GB) and harvested onto glass fiber filters. Incorporation was measured by liquid scintillation spectrometry. (b) Protein synthesis was measured by labeling 5×10^4 cells with 5 µCi [^{35}S]methionine (Amersham, > 800 Ci/mmol) for 18 h. Labeled cells were lysed with 0.5% Nonidet-P40 (NP40), 0.15 M NaCl, 50 mM Tris chloride, pH 8.0, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Aliquots of total cell lysates were spotted onto Whatman 3 MM filter paper, boiled in 10% trichloroacetic acid (TCA) for 10 min and washed in cold TCA followed by ethanol and ether. Air-dried filters were then counted in a liquid scintillation counter.

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2.3 Analysis of surface and biosynthetically labeled proteins

(a) HA1.7 was surface labeled with ^{125}I using the diphenyl glycoluril procedure (iodogen) [7]. Briefly, the cells were washed twice in PBS, resuspended in PBS to 10^7/ml and labeled with 1 mCi ^{125}I (Amersham) for 20 min. They were then washed twice in PBS and lysed in 0.5% NP40, 0.15 M NaCl, 50 mM Tris chloride, pH 8.0, 1 mM PMSF and 50 mM iodoacetamide. Cell debris was removed by centrifugation for 1 min in an Eppendorf microfuge and the cell extracts were analyzed by two-dimensional gel electrophoresis using the isoelectric focusing system described by O'Farrell [8]. (b) HA1.7 was washed once in methionine-free RPMI 1640 (Selectamine kit, Gibco) and cultured in 96-well plates at 2×10^5 cells/well in the presence of 10 μCi [^{35}S]methionine. After labeling overnight, the cells were washed once in complete medium and lysed as described above. (c) Proteins labeled using either method were analyzed by SDS-PAGE in 10–15% exponential gradients of acrylamide. After staining with Coomassie blue to locate the molecular weight markers, the gels were dried and autoradiographed using Kodak SB-5 or XAR5 film for ^{35}S and ^{125}I, respectively.

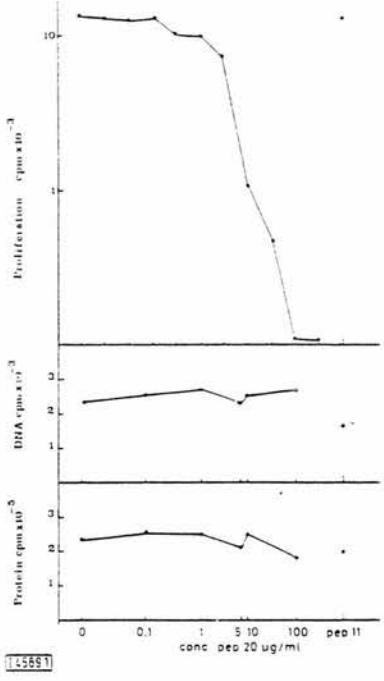

Figure 1. HA1.7 was cultured in round-bottom tissue culture wells for 18 h with [^3H]thymidine for DNA and [^{35}S]methionine for protein synthesis. The quantities of cells and isotopes used were as described in Sect. 2.2. Each point represents the mean of two determinations, with standard errors being less than 10%. The top graph indicates the incorporation of [^3H]thymidine in a standard proliferation assay after clones were treated with the indicated amounts of peptide and represents functional tolerance with p20 [2].

3 Results

3.1 Effect of p20 on synthesis of protein and DNA

To observe the effect of increased doses of p20 on macromolecular synthesis, HA1.7 was cultured under similar conditions to those described in Fig. 1, but in the presence of radioactive precursors of DNA and protein. Fig. 1 shows the ability of increased p20 concentrations to inhibit the subsequent proliferation of HA1.7 when challenged in the presence of an immunogenic concentration of p20 (1 μg/ml) and irradiated presenting cells (ref. [2], top graph). Total DNA and protein synthesis, as measured by [^3H]thymidine and [^{35}S]methionine uptake, respectively, was unaffected and remained at the same level as that of the control peptide p11.

3.2 Biosynthetic labeling of HA1.7

Although total protein synthesis in HA1.7 appeared to be unaffected by high doses of p20, it was possible that the synthesis of a small number of individual polypeptides might be altered under these conditions. Cells were therefore labeled with [^{35}S]methionine in methionine-free medium for 18 h, lysed to extract proteins from the cell membrane and cytoplasm and analyzed by SDS-PAGE. Labeling in this medium has previously been shown not to affect the ability of HA1.7 to be functionally tolerant to antigen. Fig. 2 shows the gel obtained after such a protocol. The lanes derived from cultures without peptide were duplicated to show the reproducibility of sample preparation and electrophoresis. A control peptide, p11, gave identical patterns to these zero controls (data not shown). As the peptide concentration was increased, however, several changes in the proteins synthesized became

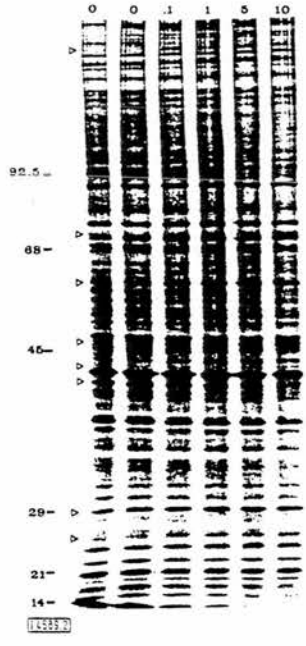

Figure 2. HA1.7 was labeled at 1×10^6/ml in 200 μl RPMI 1640 minus methionine containing 50 μCi/ml [^{35}S]methionine. p20 was added at the indicated concentrations in micrograms/ml and labeling continued for 18 h. The cells were washed once in complete medium, extracted in NP40 buffer as described in Sect. 2.3 and the entire sample was analyzed by SDS-PAGE using a 10–15% exponential gradient of acrylamide before staining and autoradiography. The molecular weight markers were: phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and lysozyme.

apparent. Thus proteins of apparent molecular masses 24, 29, 44, 47, 60, 70 and 135 kDa were enhanced, while two species of 42 and 92 kDa were diminished. Similar profiles were obtained when [^{35}S]cysteine was substituted for methionine (data not shown). Thus even relatively low levels of p20 (*e.g.* 0.1 μg/ml) were capable of enhancing the synthesis of some proteins.

3.3 Surface iodination of HA1.7

HA1.7 was cultured overnight with either 0 or 100 μg/ml of p20, followed by surface iodination with ^{125}I using the iodogen method. NP40 lysates were prepared and analyzed by isoelectric focusing followed by SDS-PAGE. Fig. 3 shows the result obtained. Some spots were induced [relative to 0 and irrelevant peptide (p11) controls] with high doses of p20, most prominently those with a molecular mass of 50, 83 and 87 kDa. Initially we had hoped to detect putative T cell receptor molecules using this technique. These have been described as having molecular masses between 80 and 90 kDa under nonreducing conditions which can be cleaved by reduction to two molecules of 30–40 and 45–50 kDa, respectively [10]. These were not observed using standard two-dimensional gels and therefore represent a very small fraction of the total surface-labeled proteins.

4 Discussion

We have examined the changes in protein synthesis which occur when cloned human T helper cells are exposed to tolerogenic doses of peptide antigen. Functional unresponsiveness is generated exclusively by specific antigen (either p20 or p11 according to the clone used) in the absence of accessory cells [2]. p20 itself has no effect on other clones for which it is not specific. In addition, modulation of the T3 antigen was shown to be peptide specific in the same way [3]. These observations would suggest that the clones "present" specific antigen to themselves in the absence of accessory cells, and that the most likely site of peptide interaction other than the specific receptors is with the HLA-DC molecules expressed on the cell surface. It is unlikely that this unresponsiveness is due to a shut off of macromolecular synthesis since the clones are still responsive to interleukin 2 (IL 2) [2]. Thus we have shown in this report that DNA and protein synthesis is unaffected by increasing doses of p20. However, examination of total surface or biosynthetically labeled proteins by SDS-PAGE revealed several changes. At present, little is known about the molecular mechanisms of lymphocyte proliferation although studies using uncloned peripheral blood lymphocytes and mitogens have indicated that new proteins are induced [11]. In that study, proteins recognized by antisera to HLA. A, B, C and DR antigens were induced, whereas the cloned T cells described here already express these antigens at the same level regardless of the amount of peptide used in preincubation as judged by phenotypic analysis [3]. In addition, the molecular weights of the induced proteins described by these investigators differ from those obtained here, although this could partly be due to differences in gel systems. We have observed the increased expression of the IL 2 receptor, detected by the anti-Tac monoclonal antibody, upon p20 stimulation [12], and it is possible that the biosynthetically labeled 60-kDa species (Fig. 2) corresponds to this receptor [13]. The newly synthesized proteins could also be cytoskeletal in origin, since receptor down regulation by internalization or shedding presumably involves some rearrangements of the cytoskeleton. Finally, some of these proteins may be regulators of gene expression although if they interact directly with nucleic acids their copy number would be expected to be very low. A definite answer to these questions can only be made when each molecule involved is identified: this will probably require screening of intracellular antigens with a battery of antisera against known lymphocyte proteins.

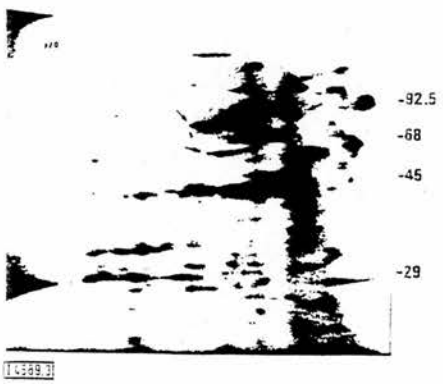

Figure 3. HA1.7 cells (2.5×10^6) were incubated with or without 100 μg/ml p20 for 18 h in RPMI 1640 containing 10% AB serum. They were then washed once in PBS and iodinated with 0.5 mCi ^{125}I using the iodogen method. After two washes with PBS the cells were lysed in NP40 buffer, made 9.5 M in urea and mixed with an equal volume of O'Farrell solution A [6]. Isoelectric focusing was performed exactly as described [6] and the gels were run in the second dimension through 10–15% gradient gels. The dried gels were autoradiographed with intensifying screens. The arrows indicate peptides enhanced upon stimulation of HA1.7 with p20.

Analysis of the cell surface proteins induced by p20 has been less revealing, since of the protein changes observed, none corresponded to the T3 and T1 antigens known to be down regulated by using serological techniques [3]. Thus the number of bands changed with high doses of p20 is clearly an underestimate.

The system we have described offer a means of reducing the complexity of the analysis of interacting cells and molecules within the immune system by using cloned cells and minimal antigenic units. It offers the additional prospect of analyzing two opposing functional states, immunity and tolerance [2]. In the initial functional studies, we found that tolerance T cells were still alive and responded to IL 2 after 7 days culture [2], suggesting that tolerance was not due to antigen-induced damage of T cells. Subsequent membrane studies confirmed that tolerance was an active state, since there was selective loss of cell surface antigens, not only the T3 complex [3]. Here we present further data that the process is complex with selectively enhanced protein synthesis. The function of the newly synthesized proteins, however, is unknown at present.

We wish to thank Dr. R. A. Lerner for the gift of peptides, Drs. Beverley and Silver for antisera, Dr. M. Contreras and colleagues at the National Blood Transfusion Centre, Edgware, for human cells and serum, Sue Smith and Jill Knight for technical assistance and Philippa Wells for typing the manuscript.

Received June 7, 1984; in revised form October 22, 1984.

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ELSEVIER

SPECIAL INVITED ASHI PAPER
David D. Eckels, Guest Editor

Differential Induction of the NF-AT Complex During Restimulation and the Induction of T-Cell Anergy

David Wotton, Julie A. Higgins, Robyn E. O'Hehir, Jonathan R. Lamb, and Richard A. Lake

ABSTRACT: Stimulation of human CD4$^+$ T-cell clones through the T-cell receptor (TcR) by high doses of specific peptide results in the induction of a long-lived state of nonresponsiveness that has been called anergy. During the induction of anergy, T cells are phenotypically similar to cells responding to an immunogenic stimulus. The amount of TcR at the cell surface is downmodulated, whereas the CD2 and CD25 receptors are increased. When restimulated, however, anergic T cells fail to upregulate transcription of the IL-2 gene and in consequence do not produce IL-2. In this study, we have compared the ability of various transcription factors to bind to their appropriate site on DNA. Factors were isolated from the nuclei of T cells that were in the induction phase of anergy or were undergoing activation. The pattern of binding activity in restimulated T cells is consistent with the pattern that has previously been shown to regulate T-cell-specific expression of the IL-2 and the β chain of the TcR genes. The measured binding to a TCF-1 site is the same in the nuclei of resting, activated, and anergized cells. The inducible factors NK-κB, β E2, CD28RC, and AP-1 are not expressed in resting cells and are twofold lower in anergized as compared with activated cells. In contrast, anergic T cells express approximately eightfold lower amounts of NF-AT, a member of the class of inducible factors that regulates IL-2 gene transcription. The failure to induce NF-AT completely may be a consequence of a diminished calcium flux, since the PKC pathway was apparently intact. It was found that the calcium ionophore ionomycin could either induce anergy or abrogate the induction of nonresponsiveness according to the dose, also suggestive of differences in calcium signaling. The pattern of expression of transcription factors during the induction of T-cell anergy is consistent with the inability of anergic cells to produce IL-2. These results demonstrate that there are differences in the early nuclear events characteristic of stimuli, the outcome of which leads to cells that are phenotypically similar, but are functionally different. *Human Immunology 42, 95–102 (1995)*

ABBREVIATIONS

APC	antigen-presenting cell
$[Ca^{2+}]_i$	intracellular calcium concentration
dT	deoxythymidine
HA	hemagglutinin
MHC	major histocompatibility complex
NF-AT	nuclear factor activated T cells
PBMC	peripheral blood mononuclear cell
PHA	phytohemagglutinin
PKC	protein kinase C
TcR	T-cell receptor

INTRODUCTION

Immunologic tolerance results from deletion of some self-reactive T lymphocytes in the thymus; other self-reactive T cells that escape deletion can be rendered nonresponsive or anergic (reviewed by Kroemer and Martinez [1]). The second process is of particular interest because the ability to render specific clones of T cells nonresponsive in vivo may contribute to attempts to control immunologically mediated disease processes. Toward this end, we have investigated the mechanisms of induction of anergy with cloned T cells in vitro. T-cell activation and interleukin-2 (IL-2) production are regulated by several signals, including those generated through the T-cell antigen receptor (TcR), protein kinase C (PKC), and changes in the intracellular free cal-

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Received (U) December 1, 1993; accepted December 6, 1993.

Human Immunology 42, 95–102 (1995)

0198-8859/95/$9.50
SSDI 0198-8859(94)00004-A

cium concentration ($[Ca^{2+}]_i$). Additionally, other costimulatory signals can act synergistically to cause synthesis of IL-2 and proliferation of the cells. The best-documented costimulatory pathway is mediated through CD28 on the T cell and the activation antigen B7/BB-1 present on the antigen-presenting cell (APC) [2–4]. Stimulation through the TcR without a costimulatory signal results in anergy [5], and under some circumstances a signal through CD28 can block the induction of anergy in murine T-cell clones [6]. For human $CD4^+$ T cells, however, stimulation through the TcR with high doses of appropriate peptide, in either the presence or absence of APCs, will inhibit subsequent antigen-dependent proliferation [7]. These experiments clearly show that the optimum dose of peptide for the induction of anergy is higher in the presence of APCs. Thus, it seems that the magnitude of the signal received through the TcR can override an otherwise immunogenic stimulus and induce anergy. Fully activated T cells can also be rendered nonresponsive [8]. And these observations anticipate clinical intervention in immunologically mediated diseases, where the target T cells are more likely to be activated than in a resting state and the T cells will certainly be in the presence of APCs.

The phenotype of T cells during the induction phase of anergy is similar to the phenotype of cells undergoing stimulation. Key changes at the cell surface include downmodulation of the TcR complex and upregulation of CD2 and CD25 receptors. Interestingly, signals through the TcR upregulate transcription of the β chain of the TcR gene [9], whereas transcription of the CD2 gene is not inducible. However, a complex array of post-transcriptional mechanisms regulates TcR gene expression [10], such that the overall effect of activating or anergizing signals is downmodulation of cell surface TcR. After an activating signal, T cells upregulate their expression of the CD28 antigen. In contrast, T cells that receive a high dose of specific peptide as a tolerizing signal do not [8, 11]. A signal through CD28 is a pleiotropic event: it is costimulatory for T-cell proliferation and results in the upregulation of several T-cell-derived cytokines by stabilizing their mRNAs [12]. Ligation of CD28 affects IL-2 synthesis by the generation of a nuclear complex (CD28RC) that increases transcription from the IL-2 gene promoter [13]. The induction of the CD28RC is, however, clearly not specific for signals through the CD28 receptor as the complex is induced by the mitogenic combination of PMA and anti-CD3 antibodies [14].

In this report, we have analyzed the effect of activating and anergizing regimens on the induction of some selected transcription factors that regulate expression of the β chain of the TcR and the IL-2 gene. These factors are well characterized and both T-cell-specific and general transcription factors are represented. The IL-2 promoter contains binding sites for nuclear factors including NF-AT, Oct, NF-κB, and AP-1, which are all potentially sensitive to activation of PKC. Members of a family that include the nuclear oncogenes *Fos* and *Jun* bind as a heterodimeric complex to an AP-1 DNA-binding site. Dimerization occurs by the interaction of leucine zipper domains in the two proteins and is a prerequisite for DNA binding. The AP-1 site is the major target of transcriptional induction after PKC activation in the IL-2 gene promoter [15].

NF-AT is an inducible factor that is restricted to T cells and is also a major regulator of IL-2 gene transcription. NF-AT is formed when a preexisting cytoplasmic subunit translocates to the nucleus and combines with newly synthesized Fos and Jun proteins [16]. FK506 and cyclosporin A block translocation of the cytoplasmic component without affecting synthesis of the nuclear subunit [17], suggesting that the translocation is a calcium-dependent event. In nonstimulated cells, NF-κB resides in the cytoplasm in an inactive complex with the inhibitor I-κB. Stimulation causes release of I-κB and allows NF-κB to enter the nucleus, bind to DNA control elements and, thereby, aid transcription. Activation is triggered by a variety of agents including IL-1, TNF, and phorbol esters. The TcR β-chain enhancer responds to PKC-mediated activation signals through a functional domain that includes the βE2 element [18]. Multimerized βE2 can act in isolation as a phorbol-ester-responsive element; it contains a consensus Ets-binding site and binds directly to the product of the c-ets-1 protooncogene. βE2 can also bind a second family of nuclear factors, the core-binding factors [9]. TCF-1 is a noninducible T-cell-specific transcription factor: it is known to bind within the CD3 ϵ enhancer and in the TcR α enhancer. It shares a region of homology with other transcription factors termed the high-mobility group 1 (HMG) box [19].

MATERIALS AND METHODS

Peptides. The influenza hemagglutinin peptide (residues 307–319; HA 307–319) was synthesized using standard solid-phase technology and further purified by reverse-phase HPLC. The constitution of the peptide was confirmed by amino acid analysis as previously described [20].

Antibodies. Flow-cytometric analysis was performed using fluorescein-conjugated murine monoclonal antibodies. Anti-Leu4 (CD3), anti-IL-2 receptor (CD25), and a mouse IgG_1 control were purchased from Becton Dickinson (Oxford, UK). The murine anti-CD3 antibody used for cell activation was a kind gift from H. Spits

(The Netherlands Cancer Research Institute, Amsterdam).

Cloned T lymphocytes. The isolation of HA1.7, a clone of T cells reactive with HA 307–319 has been reported in detail previously [7]. Briefly, peripheral blood mononuclear leukocytes (PBMCs) were stimulated with purified HA 307–319 in RPMI-1640 (Gibco Laboratories, Uxbridge, UK) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 5% human AB$^-$ serum. The activated T cells were cloned by limiting dilution in the presence of irradiated autologous PBMCs, IL-2 (10% Lymphocult T; Biotest Folex, Frankfurt; i.e. 10 U/ml) and antigen. Growing HA1.7 cells were expanded by stimulation with antigen and filler cells every 7 days. After stimulation, the cells were expanded with 10% Lymphocult T every 3–4 days. The cells were rested 7–8 days after the last addition of filler cells and antigen prior to their use in experiments. Resting cloned T cells (2×10^5/well) were activated by incubation with anti-CD3 antibody insolubilized on tissue culture plates (12 μg/ml) together with 10% Lymphocult T. To induce anergy, T cells were incubated with HA 307–319 (50 μg/ml). In some experiments, the calcium ionophore, ionomycin (0.2–1 μg/ml) was added. After 24 hours, the cells were pooled and washed three times. The majority were used to make nuclear extracts, whereas some were assessed for their ability (2×10^4/well) to respond to an immunogenic challenge: either by APCs (irradiated histocompatible [DR1$^-$] PBMCs 2×10^4/well) and antigen (HA 307–319, 0.3 μg/ml) or by incubation with anti-CD3 antibody insolubilized on tissue culture plates (12 μg/ml).

T-cell proliferation assays. After a further 60 hours of incubation [^{3}H]methyl thymidine (1 μCi/well; Amersham International, Amersham, UK) was added and the cultures harvested 8–16 hours later. Proliferation as correlated with [^{3}H]methyl thymidine incorporation was measured by liquid scintillation spectroscopy. The results are expressed as mean counts per minute (cpm) for triplicate cultures.

Flow cytometry. T cells (3×10^5) were stained directly by using saturating concentrations of fluorescein-conjugated murine monoclonal antibodies. Forward and side scatter was used to identify viable cells. Cell populations were analyzed by flow cytometry using an Epics-Profile II (Coulter, Luton, UK). Data are expressed as the channel number representing mean fluorescence intensity.

Oligonucleotides. Oligonucleotides were synthesized using an Applied Biosystems DNA synthesizer and purified using OPC (oligonucleotide purification columns, Applied Biosystems), according to the manufacturers' general instructions. The sequences used were

βE2:	5′-GATCCACAACAGGATGTGGTTTGACATTTA-3′
NF-AT:	5′-GATCTAAGGAGGAAAAACTGTTTCATCG-3′
NF-κB:	5′-GATCCAGTGGGAAATTCCTCG-3′
TCF-1:	5′-GATCCTGGGAGACTGAGAACAAAGCGCTCTCACACGGGA-3′
CD28RC:	5′-GTCTGATGACTCTTTGGAATTTCTTT-3′
AP-1:	5′-GATCTATCTCTGAGTCAATCAGCG-3′

The reverse and complement of each sequence was also synthesized with a noncomplementary 5′ dG and without the 3′dC.

Electrophoretic mobility-shift assay. Nuclear extracts were prepared by a rapid method as described by Dignam et al. [21], with several modifications. Cells (2×10^6) were washed in ice cold PBS, pelleted and resuspended in lysis buffer (10 mM Hepes pH 7.9, 1.5 mM $MgCl_2$, and 10 mM KCl). The mixture was vortexed and left on ice for 5 minutes. Cell nuclei were pelleted by centrifugation and resuspended in glycerol buffer (25% vol/vol glycerol, 20 mM Hepes pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, and 0.2 mM EDTA). The nuclear lysate was left on ice for 1 hour with occasional vortexing. The nuclear debris was pelleted and the extract retained. Protein concentrations were estimated using the Biorad protein assay kit. All buffers contained 0.5 mM PMSF, 1.0 mM benzamidine, 5 μg/ml aprotinin, 5 μg/ml pepstatin, 30 μg/ml leupeptin, and 0.5 mM DTT.

Oligonucleotides were radiolabeled with ^{32}P [dCTP] by filling in a dG overhang using AMV-RT, and the probes were then purified by separation on 7.5% polyacrylamide gels. Binding reactions contained 20 μg acetylated BSA, 2 μg poly(dI–dC) · poly(dI–dC), 0.5 μg salmon sperm DNA, 0.005% NP40, 5 mM $MgCl_2$, 80 mM NaCl, 10 mM Hepes pH 7.9, 2.5 μg of nuclear extract, and 0.5 ng of probe with an activity of around 10,000 cpm. Oligonucleotide competitors were preincubated with the nuclear extract for 5 minutes before the addition of probe. Nonspecific oligonucleotides were used in the final analysis as an indication that complexes were not being retarded through a general capacity to stick to DNA. In each case, the nonspecific oligonucleotide was TCF-1, except in the TCF-1 gel shift, where the AP-1 oligonucleotide was used. Reactions were incubated at room temperature for 20 minutes and then separated on 5% polyacrylamide gels. Gels were dried for autoradiography or were quantitated directly from the dried gels by using an Ambis scanner.

RESULTS

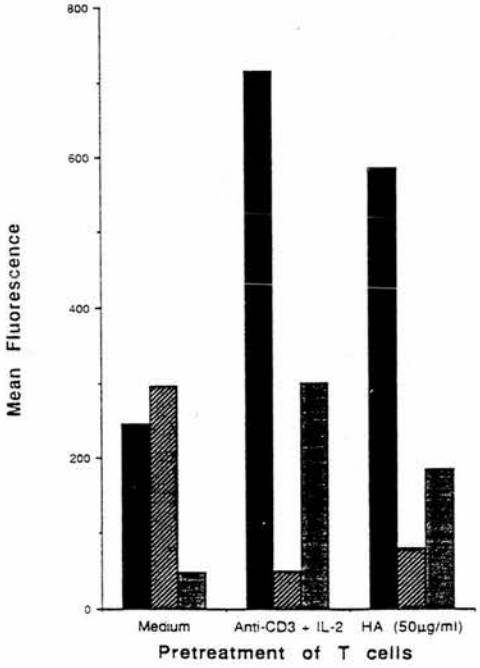
Phenotypic changes during activation and the induction of anergy. The phenotypic modulation of the HA 307–319-reactive CD4$^-$ T cells (HA1.7) after treatment with

activating and tolerizing regimens has already been reported in detail [22]. T cells were rested for 7 days after the last cycle of expansion and then activated with anti-CD3 antibody and 10% Lymphocult T, or exposed to a tolerizing concentration of HA 307–319. Cells were analysed by fluorescence flow cytometry to determine the level of surface CD3, CD25, and CD2 (Fig. 1). Consistent with our previous findings, both treatments resulted in cells that had markedly downregulated membrane CD3 expression. In contrast, IL-2 receptor (CD25) and CD2 expression were upregulated.

Induction of T-cell anergy by treatment of cells with HA 307–319. The T cells were tested for their ability to respond to specific peptide, 24 hours after receiving an activating or anergizing signal (Fig. 2). The data show that preactivation enhances the ability of cells to proliferate in response to an immunogenic challenge when compared with cells incubated in medium alone. This phenomenon is probably due to the induction of CD25 and the manifest hyperresponsiveness of activated cells to IL-2 (Fig. 2). After incubation with HA 307–319 (50 μg/ml) for 24 hours, the T cells failed to proliferate when restimulated with HA 307–319 presented in an immunogenic form (Fig. 2). The addition of exogenous IL-2 caused proliferation of the anergized cells, confirming functional inactivation was not the result of cytolysis.

Differential expression of the NF-AT complex in activated and tolerized T cells. Nuclear extracts were incubated with radiolabeled double-stranded DNA probes that contained known *cis*-acting sequences to determine which transcription-factor-binding activities were expressed after treatment of cells with activating or tolerizing stim-

FIGURE 1 Phenotypic modulation of T cells exposed to activating and anergizing signals. Membrane expression of CD2 (*solid bars*), CD3 (*cross-hatched bars*), and CD25 (*stippled bars*) of T cells exposed to different conditions were compared by flow cytometry at 24 hours after stimulation.



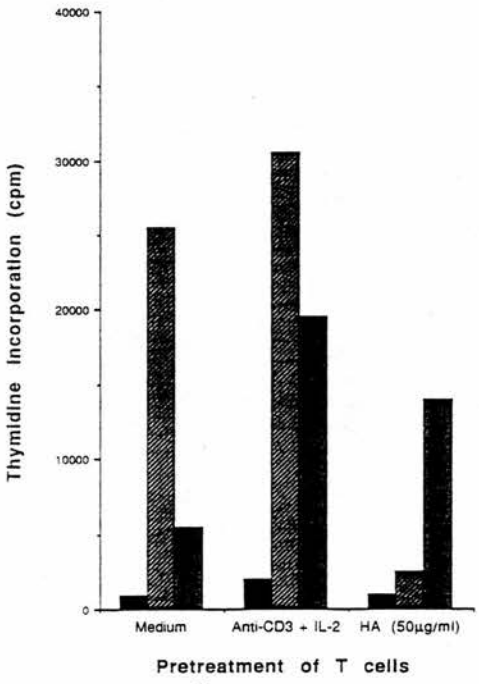


FIGURE 2 The ability of specific peptide to induce anergy in activated T cells. Cloned T cells (HA1.7; 10^6/ml) were pretreated by culturing with insolubilized anti-CD3 antibody and IL-2, with HA 307–319 at 50 μg/ml or in medium alone for 24 hours. After pretreatment, the T cells (10^5/ml) were assayed for their ability to respond to restimulation with HLA-DR1 + APCs (irradiated PBMCs) alone (*solid bars*), HA 307–319 (0.3 μg/ml) + APCs (*cross-hatched bars*), or IL-2 alone (*stippled bars*). Proliferation as correlated with [^{3}H]methyl thymidine incorporation was determined 72 hours later.

uli. Two independent characteristics divide the factors into four groups, three of which were chosen for study: T-cell-specific inducible factors include NFκB, NF-AT, βE2, and the CD28RC; AP-1 is a general and inducible molecular complex, whereas TCF-1 [19] is an example of a T-cell-specific noninducible factor. TCF-1 and βE2 are involved in the regulation of transcription of the β chain of the TcR, whereas the others have a role in transcription of the IL-2 gene. As expected, the noninducible factor, TCF-1, is the only factor clearly present in the nuclei of untreated cells and its concentration does not vary in activation and anergy (Fig. 3). All six probes formed complexes with extract from activated cells that were competed by specific, but not by nonspecific, oligonucleotide competitors. The lower band in the NF-κB shift is a nonspecific complex, whereas the upper is authentic NF-κB. Equal amounts of protein were used from each nuclear lysate, and this is substantiated by the observation that binding to the TCF-1 site is the same in the nuclei of resting, activated, and anergized cells. Differences in the pattern of induction of specific transcription factors were quantified using an Ambis scanner; radioactivity was measured directly from the dried gels. These results confirm a relative failure of anergic T cells to upregulate NF-AT (Table 1). The inducible factors NF-κB, βE2, CD28RC, and AP-1 are not expressed in resting cells, and are 1.6- to 2.2-fold lower in anergized as compared with activated cells. In contrast, anergic T cells express approximately eightfold lower amounts of NF-AT than cells that received an activating signal.

Functional inactivation in the presence of calcium ionophore. The failure to establish good NF-AT expression in the induction phase of anergy suggested that these T cells may be missing some biochemical aspect of an activating signal. The observation that AP-1 and βE2 binding were relatively well induced in anergized T cells suggested that the pathway through PKC was intact. There is an approximate fourfold difference between the induction of AP-1 and NF-AT when activated cells are compared with anergized cells, and both of these treatments involve stimulation through the TcR. Since NF-AT consists of AP-1 plus a second component that translocates to the nucleus as a result of calcium-dependent

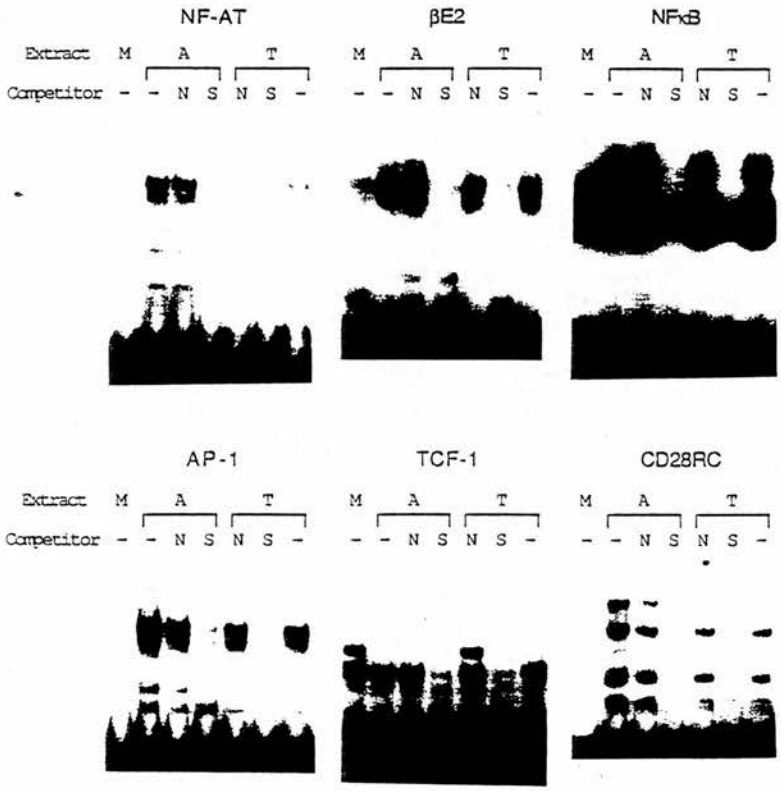


FIGURE 3 Electrophoretic mobility shift assay. Nuclear extracts were prepared from resting T cells incubated in medium alone (*M*), and from activated (*A*) or tolerized (*T*) cells. Double-stranded DNA probes (as indicated) were incubated with the extracts in the presence of 100-fold excess of either a specific (*S*) or nonspecific (*N*) oligonucleotide competitor and separated on polyacrylamide gels.

TABLE 1 Ratio of transcription factor binding activities in activation and anergy

Factor	Ratio[a] activated:anergized
NF-AT	8.1:1
βE2	1.8:1
NF-κB	1.6:1
AP-1	2.1:1
CD28RC	2.2:1
TCF-1	1.0:1

[a] Retarded complexes were quantified directly from the dried gel using an Ambis scanner.

phosphorylation, it seemed logical to investigate the Ca^{2+} pathway further. It is noteworthy in this regard that both core-binding factor (CBF) and the ets transcription factors bind to the βE2 element: ets is inducible whereas CBF is not [9]; this explains the small shift of the βE2 element when using the extract from resting T cells (Fig. 3). βE2 activity is inducible principally through PKC and not the Ca^{2+} pathway [23], thus providing further evidence that the PKC pathway is relatively intact during the induction of anergy. T cells were therefore treated with calcium ionophore at the time that they received an anergizing dose of peptide to establish whether an additional calcium flux could abrogate the induction of T-cell anergy. T cells were restimulated with either insolubilized anti-CD3 or HA 307–319 presented on irradiated histocompatible ($DR1^-$) PBMCs. The results (Table 2) show that ionomycin itself can induce anergy in a dose-dependent manner, but that peptide-induced anergy is abrogated only by a low dose of ionomycin. This applies both to restimulation with peptide and APCs and with anti-CD3.

DISCUSSION

Several groups have developed systems to study T cell anergy in vitro. Each method includes a signal through the TcR, though this can be delivered specifically, for example with peptides, or nonspecifically, using anti-CD3 antibodies or lectins. T-cell proliferation is augmented by costimulatory signals, and anergy can be induced in murine T cells by antigen receptor stimulation in the absence of costimulation. The costimulatory molecule B7/BB-1 is functionally inactivated by a chemical fixation process, and the use of such cells to present antigen results in anergy. This mechanism does not result in the phenotypic changes that characterize high-dose peptide anergy of human T cells, but the cells have in common a reduced ability to release IL-2 on rechallenge. Go and Miller examined the expression of a series of transcription factors after stimulation of murine T-cell clones with normal and with chemically modified APCs [24]. The tolerogenic stimulus induced less NF-AT and lower amounts of one of the two NF-κB-binding factors. The AP-1 transcription factor was induced and was not obviously differentially regulated. These results are in very close accord with the results presented here, but differ markedly from a third independent study [25]. This group showed that anergic T cells, generated by exposing the cells to concanavalin A, did not make IL-2. However, this was associated with a specific downregulation of the AP-1 complex. These experiments showed no differences in the NF-AT complex, a surprising finding since NF-AT contains AP-1. Collectively, these data indicate that the control of IL-2 gene expression during anergy induction and during normal stimulation of anergized cells is distinct, and suggest the presence of additional regulatory elements that control synthesis and release of IL-2.

TABLE 2 Proliferative capacity of the human T-cell clone HA 1.7 after various pretreatments

Pretreatment	Restimulation[a]				
	APC[b]	APC + peptide[c]	IL-2	Anti-CD3	Anti-CD3 + IL-2
Medium	109 (22)	17,959 (4)	12,855 (7)	3422 (18)	22,565 (5)
HA50[d]	1041 (30)	8250 (34)	27,620 (23)	1084 (30)	33,502 (4)
HA50 + I[e] (1 μg/ml)	616 (31)	1770 (9)	20,936 (12)	441 (24)	24,317 (3)
HA50 + I[e] (0.2 μg/ml)	7254 (11)	18,344 (10)	34,092 (12)	6774 (21)	45,441 (7)
I[e] (1 μg/ml)	167 (34)	1602 (9)	14,313 (12)	212 (38)	17,286 (10)
I[e] (0.2 μg/ml)	105 (19)	6033 (12)	13,183 (6)	1768 (10)	20,557 (10)
Anti-CD3	202 (58)	16,896 (55)	13,306 (8)	7270 (24)	30,651 (14)

[a] Cells were restimulated 24 hours after pretreatment and proliferation as correlated with [^{3}H]methyl thymidine incorporation was determined 72 hours later in duplicate. The numbers in parentheses refer to SEM.

[b] APC, irradiated DR1 + PBMC.

[c] Peptide, HA 307–319 (0.3 μg/ml).

[d] HA50, HA 307–319 (50 μg/ml).

[e] I, ionomycin (concentration as shown).

In an interesting series of experiments designed to investigate the relationship between NF-AT and gene expression, the *lacZ* gene was placed under the control of tandem copies of the NF-AT-binding site in transfected T cells. Following exposure of the cloned stably transfected T cells to TcR-specific stimuli, a bimodal pattern of gene expression was noted. Increasing concentrations of the stimulus increased the fraction of *lacZ*$^+$ cells, but not the level of *lacZ* activity per cell [26]. The pattern of expression was not dependent upon cell-cycle position or heritable variation. And the results, therefore, suggest that the concentration of NF-AT must exceed a critical threshold before transcription of a linked gene is induced. Other transfected T cells, in which *lacZ* is controlled by NF-κB or the entire IL-2 promoter, also show bimodal expression patterns after stimulation [27]. It seems likely then that transcription factors have concentration thresholds below which they cannot initiate transcription of linked genes. A completely different phenotype might, therefore, occur with only a partial loss of an inducible factor.

T-cell anergy is mediated, at least in part, by a loss of the costimulatory pathway, and the CD28 receptor is only one component of a complex system regulating T-cell activation. There are several surface molecules that transmit costimulatory signals, and these might substitute if the CD28 pathway were blocked. For example, superantigen-induced proliferation of human T cells is not dependent on costimulation through CD28, but is affected by interactions between the CD11a–CD18 complex and its counter receptors ICAM1–3 [28]. The results reported here show that the induction of T-cell anergy, when compared with T-cell stimulation, is characterized by a reduced expression of the NF-AT complex. NF-AT is induced in T cells stimulated through the TcR and is required for IL-2 gene induction. The induction of NF-AT probably requires two activation-dependent events: the translocation of a preexisting cytoplasmic component and the synthesis of a nuclear component. The newly synthesized nuclear component of NF-AT is the transcription factor AP-1, and it contains the Fos and Jun proteins [16], whereas the translocation depends on dephosphorylation by calcineurin [29]. Our results show no primary failure in the ability of tolerized cells to produce AP-1; it therefore seems likely that the difference lies in the ability of these cells to translocate the cytoplasmic component of the complex to the nucleus. Maximal expression of NF-AT in T cells requires at least two signals: triggering of the TcR in association with either PKC activation or a $[Ca^{2+}]_i$ flux. Activation through the TcR has many downstream biochemical effects, and signals other than a calcium flux or PKC can regulate NF-AT expression in peripheral blood-derived T cells [30]. The TcR can regulate multiple intracellular signals in T cells, and the biochemical events following ligation of the receptor may depend upon ligand density as well as the nature of additional signals. We have shown that the combination of these signals, generated by the addition of ionomycin during the induction of anergy, leads to a different end state according to the concentration of ionomycin. And this suggests that the triggering of particular downstream events is sensitive to different thresholds of $[Ca^{2+}]_i$.

Molecules associated with the induction of anergy (anergens) are likely to be a diverse group that may affect processes in distinct cellular compartments. Induction of anergy is an active process that requires protein synthesis [22]. Therefore, some anergens will be newly synthesized molecules and others may be activated by phosphorylation or translocated to an active site. While this study has not directly identified any potential anergen, the observation that the amount of NF-AT available in the nucleus for transcription is different in activation and anergy shows that the cells have taken alternative developmental steps.

ACKNOWLEDGMENTS

This work was supported by the Medical Research Council (MRC) and Wellcome Trust. R.E.O. is a Wellcome Senior Clinical Research Fellow. The authors express their gratitude to Mark Larché and Alex Faith for helpful discussions concerning the manuscript.

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2.3 ANTIGEN MEDIATED MODULATION OF T CELL FUNCTION

2.3.4 INDUCTION OF PERIPHERAL TOLERANCE *IN VIVO*

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Brief Definitive Report

Inhibition of T Cell and Antibody Responses to House Dust Mite Allergen by Inhalation of the Dominant T Cell Epitope in Naive and Sensitized Mice

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Summary

Antigen-specific CD4+ T cells play an important role in the allergic immune response to house dust mite (HDM) allergens in humans. The group 1 allergen of *Dermatophagoides* spp. is a major target antigen in both B and T cell recognition of HDM. In vitro studies have shown that the presentation of peptides to human T cells under appropriate conditions may lead to a state of specific nonresponsiveness. Therefore, to determine if peptides are able to modulate the function of allergen-reactive T cells in vivo, we have used a murine model of T cell recognition of the HDM allergen Der p 1. The results demonstrate that inhalation of low concentrations of peptide containing the major T cell epitope of Der p 1 (residues 111–139), induces tolerance in naive C57BL/6J mice such that they become profoundly unresponsive to an immunogenic challenge with the intact allergen. When restimulated in vitro with antigen, lymph node T cells isolated from tolerant mice secrete very low levels of interleukin 2, proliferate poorly, and are unable to provide cognate help to stimulate specific antibody production. Furthermore, intranasal peptide therapy was able to inhibit an ongoing immune response to the allergen in mice and this has potential implications in the development of allergen-based immunotherapy.

Approximately 10% of the population suffers from allergy to house dust mite (HDM) and CD4+ T cells are known to play an important role in allergic sensitization (1). In vitro studies have shown that human HDM-reactive CD4+ T cell clones can be rendered unresponsive by exposure to either high doses of their specific peptide epitope or to superantigens (2–6). Therefore, by modulating the function of allergen-reactive T cells in vivo it may be possible to control an established allergic immune response in humans. Independent studies have shown that immunogenic peptides can preferentially induce antigen-specific T cell tolerance in naive mice when administered either intraperitoneally in IFA or intravenously (7–9). Experimental animals may also be rendered immunologically tolerant by feeding or inhaling antigens, and this state of nonresponsiveness is usually long-lasting and antigen specific (10–13). Experimental allergic encephalomyelitis (EAE) is a demyelinating autoimmune disease of rodents, and CD4+ T cells specific for myelin basic protein (MBP) are responsible for the development of disease. Rats can be protected from developing EAE by oral administration of MBP before an encephalitogenic challenge from the autoantigen (14, 15). Similarly, susceptible $H2^u$ mice can be protected from developing EAE by inhalation but not oral administration of a peptide containing the immunodominant T cell epitope on MBP (Ac 1–9, 1–11) (16). In a previous study, we have shown, using a murine model, that T cell responses to the Der p 1 allergen were inhibited by feeding recombinant peptides containing either major or minor T cell epitopes (Hoyne, G. F., M. G. Callow, M.-C. Kuo, and W. R. Thomas, manuscript submitted for publication). The induction of tolerance resulted in decreased responses in LN T cells to the whole antigen in vitro, and a loss of reactivity to all T cell epitopes on the antigen was observed.

In this study, we show that if naive C57BL/6J mice inhale small quantities of synthetic peptides containing T cell epitopes, they become profoundly unresponsive to a powerful immunogenic challenge with the allergen. LN T cells from tolerized mice secrete low levels of IL-2 and proliferate poorly when restimulated with antigen. They are also unable to provide help for antibody production in vitro. Furthermore, in-

 J. Exp. Med. © The Rockefeller University Press · 0022-1007/93/11/1783/06 $2.00
Volume 178 November 1993 1783–1788

tranasal peptide therapy inhibited T cell responses in mice that had been previously sensitized to the Der p 1 allergen. These studies indicate that administering immunogenic peptides intranasally may be an effective way to control aberrant immune responses that give rise to specific disease states in humans.

Materials and Methods

Animals. Inbred C57BL/6J mice were purchased from the Animal Resource Centre (Murdoch, Western Australia) at 6–8 wk of age and were kept under specific pathogen-free conditions.

Antigens. The house dust mite allergen Der p 1 was affinity purified from spent mite medium (17). Synthetic peptides derived from the Der p 1 sequence were synthesized using standard F-moc chemistry, peptides were purified by reverse-phase HPLC, and the sequence of individual peptides were confirmed by sequencing. The peptides used in this study were p1 111–139, p1 156–168 of the group 1 allergen of *Dermatophagoides pteronyssinus* (Der p 1) and p2 21–35 from the group 2 allergen (Der p 2).

Preparation of Recombinant Proteins. A cDNA insert encoding the 57–130 fragment of the Der p 1 protein was ligated to the pGEX vector which expresses the fusion proteins with glutathione-S-transferase in *Escherichia coli*. (18). The procedures for the cloning and expression of the fragments have been described (19). Log phase *E. coli* cells transformed with the pGEX-based construct were induced to express the recombinant protein by adding 0.1 mM isopropylthiogalactosidase (Promega Corp., Madison, WI). The fusion protein was purified to homogeneity using glutathione-coupled agarose (Sigma Chemical Co., St. Louis, MO) following the techniques previously described by Smith and Johnson (18), and eventually lyophilized.

Induction of Intranasal Tolerance. Mice were lightly anesthesized under ether and peptides dissolved in PBS were administered intranasally in a total volume of 10 μl using a micropipette on three consecutive days. Mice were immunized subcutaneously at the base of tail 7 d after the last treatment with 100 μg of Der p 1 emulsified in CFA (Difco, Detroit, MI) in a volume of 0.2 ml. To inhibit ongoing responses, mice were immunized intraperitoneally with Der p 1 in CFA, and 10 d later received 100 μg of p1 111–139 intranasally on five consecutive days. Mice were then reimmunized 7 d later with 100 μg of Der p 1 in IFA (Difco) subcutaneously at the base of tail.

Culture Medium. LN cells were cultured in DME (GIBCO BRL, Gaithersburg, MD) supplemented with 2% FCS (Commonwealth Serum Laboratories [CSL], Parkville, Victoria, Australia), 50 μM 2-ME (Sigma Chemical Co.), 2 mM L-glutamine (CSL), and 20 μg/ml gentamycin (David Bull Laboratories, Mulgrave, Victoria, Australia). CTLL-2 cells were maintained in RPMI (GIBCO BRL) plus 10% FCS.

T Cell Assays. The periaortic and inguinal LNs were expressed through a stainless steel wire mesh, washed, and cultured at 4×10^5 cells in a volume of 0.2 ml in culture medium in a 96-well flat-bottom tissue culture plate (Becton Dickinson Labware, Lincoln Park, NJ). Protein or peptide antigens were added at various concentrations and the cells were incubated at 37°C for 24 h. Supernatants were collected and stored at −20°C until required for the assay.

Lymphokine Assays. The CTLL-2 cell line proliferates maximally with IL-2 but only poorly in the presence of IL-4 (20). 5×10^3 CTLL-2 cells per well were cultured in supernatants for 24 h at 37°C and pulsed with 1 μCi of [^{3}H]thymidine. Cells were harvested onto glass fiber filters and the proliferation determined by incorporation of [^{3}H]thymidine which was assessed using a direct beta counter (Packard Matrix 9600; Packard Instruments, Meriden, CT).

In Vitro Antibody Production. Mice were immunized with 100 μg of Der p 1 in CFA and 10 d later, the spleens of two mice were pooled and RBCs were lysed with sterile distilled water. Periaortic and inguinal LN cells were isolated 7 d after immunization from mice that had been treated on three consecutive days with either PBS (control) or p1 111–139 (tolerant) intranasally and immunized subcutaneously 7 d later with Der p 1/CFA. 2×10^6 spleen or LN cells were cultured alone in the presence of Der p 1 in a volume of 1 ml culture medium in a 24-well tissue culture plate. Alternatively, 10^6 spleen cells were mixed with 10^6 LN cells from control or tolerant mice and cultured in a 1-ml volume with the addition of antigen. Cells were cultured for 7 d at 37°C and the supernatants were assayed for the presence of Der p 1-specific Abs using an ELISA.

ELISA. Affinity-purified protein was diluted in 50 mM Na HCO_3 buffer, pH 9.6, and coated onto an ELISA plate at 100 μg/ml in a volume of 50 μl/well. The plate was incubated overnight at 4°C and the next day was washed three times with PBS plus 0.05% Tween 20. Nonspecific binding sites were blocked by incubating the plate with a solution of PBS containing 1% BSA for 1 h at room temperature. After three washes, serum was added to the plate at appropriate dilutions and incubated for 2 h at room temperature. The plate was washed as described and a 1/1,000 dilution of goat anti-mouse Ig conjugated to horseradish peroxidase was added and incubated for 1 h at room temperature. After a final wash, the color was developed with ABTS containing 1% hydrogen peroxide for 20 min at room temperature. The reaction was stopped by addition of 0.1 M citric acid plus 0.01% sodium azide, and the OD of the plate was measured at 405 nm using an ELISA plate reader. Results are expressed as ELISA OD units.

Results and Discussion

C57BL/6J mice are high responders to the HDM allergen Der p 1 and T cells recognize three different epitopes that are located within the following sequences: 110–131, 78–100, and 21–49 (Hoyne, G. F., et al., manuscript submitted for publication). The ability of purified recombinant GEX p57–130 and a synthetic peptide that contains the immunodominant determinant, p1 111–139 to induce tolerance when administered intranasally were compared. This epitope was chosen for study because it represents a major T cell epitope recognized by human Der p 1-specific T cell clones (6). Mice received 100 μg of either peptide or PBS intranasally on three consecutive days and 1 wk later, all mice were challenged with Der p 1 in CFA. LN cells draining the site of injection were collected 7 d later and stimulated in vitro with either protein or peptide for 24 h and the supernatants were assayed for IL-2. LN cells from mice treated with either p1 111–139 or GEX p57–130 failed to respond with either Der p 1 (Fig. 1 *A*) or the T cell epitopes p1 111–139, p1 78–100 and p1 21–49 (Fig. 1, *B–D*). Control mice that received PBS intranasally developed strong IL-2 responses to both the intact protein and the peptides (Fig. 1).

In addition, mice received either PBS or p1 111–139 intranasally and 1 wk later were challenged subcutaneously with an immunogenic concentration of p1 111–139. LN cells were cultured in vitro with p1 111–139 and IL-2 secretion and T

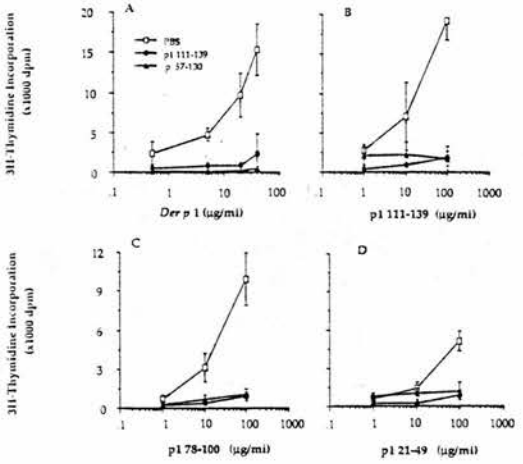

Figure 1. Peptides given intranasally to mice can inhibit T cell responses. Mice were treated with either PBS (□), 100 μg of GEX p57–130 (▲), or p1 111–139 (●) intranasally on three consecutive days and 1 wk later all mice were immunized with 100 μg of Der p 1/CFA. LN cells were collected 7 d later and cultured in vitro with (*A*) Der p 1 protein, (*B*) p1 111–139, (*C*) p1 78–100, or (*D*) p1 21–49 for 24 h. Data shows the mean IL-2 response of five mice per group ± SD.

cell proliferation were determined. LN cells from control mice proliferated strongly to the peptide in vitro and produced IL-2 in a dose-dependent manner. However, T cells from peptide-treated mice proliferated only poorly and secreted only very low levels of IL-2 when rechallenged with the peptide in vitro (data not shown). Thus the induction of tolerance results in a state of nonresponsiveness to the whole antigen as a result of the failure of antigen-specific T cells to secrete IL-2 and to proliferate when restimulated. Tolerance to the immunodominant epitope also downregulated the response of T cells directed to other epitopes on the antigen. Recently it has been reported that susceptible $H2^u$ mice are protected from developing EAE by inhalation of a synthetic peptide containing the immunodominant epitope MBP Ac 1–9 or Ac 1–11 before encephalitogenic challenge with MBP or peptide (16). In this study (16), the authors also observed that after the induction of tolerance to Ac 1–9 T cell responses to subdominant epitopes on MBP were equally downregulated.

To determine the concentration of peptide required to induce peripheral tolerance, mice were treated intranasally on three consecutive days with either PBS or 1, 10, or 100 μg of p1 111–139 and then challenged 1 wk later with Der p 1 in CFA. LN cells were collected 7 d after immunization and cultured in vitro with either Der p 1 or p1 111–139. T cell responses from each of the test groups after in vitro stimulation with Der p 1 or p1 111–139 were markedly downregulated (Table 1), whereas T cells from control mice secreted large amounts of IL-2 when stimulated in vitro with either Der p 1 or peptide (Table 1). The immunodominant peptide at concentrations as low as 3 × 1 μg induced peripheral tolerance when given intranasally. It is interesting to note that inhalation of similar levels of an intact protein in mice can induce immunological tolerance (12). Therefore mucosa of the respiratory tract appears to provide a highly efficient route for the induction of tolerance.

Mice tolerized with p1 111–139 also failed to produce specific Abs. To demonstrate this, spleen cells collected from Der p 1 immune mice were cultured in vitro either alone or with LN cells from mice that had been treated intranasally with PBS or p1 111–139 and immunized subcutaneously with Der p 1 in CFA. Lymphocytes from both the test and control groups were cultured with Der p 1 for 7 d and the presence of anti-Der p 1 Abs in the culture supernatants were examined by ELISA. Der p 1-specific Abs were present in the supernatants of both immune spleen cells and LN cells from PBS-treated mice, but no Abs were detected in the superna-

Table 1. *Intranasal Tolerance Induced with Microgram Quantities of Peptide*

	Intranasal dose of antigen			
	PBS	3 × 1 μg	3 × 10 μg	3 × 100 μg
In vitro antigen				
Der p 1 (40 μg/ml)	5,075 (± 1,354)	890 (± 314)	1,088 (± 391)	827 (± 321)
p1 111-139 (100 μg/ml)	10,698 (± 3,477)	1,671 (± 692)	999 (± 643)	773 (± 537)
Medium	241 (± 62)	328 (± 139)	560 (± 298)	401 (± 537)

C57BL/6J mice were treated on three consecutive days with p1 111–139 intranasally with the doses indicated or with PBS. 1 wk later mice were immunized with Der p 1 in CFA and the periaortic and inguinal LN were collected 7 d later and cultured in vitro with either Der p 1 or p1 111–139. Data shows the mean response of five mice per group ± SD.

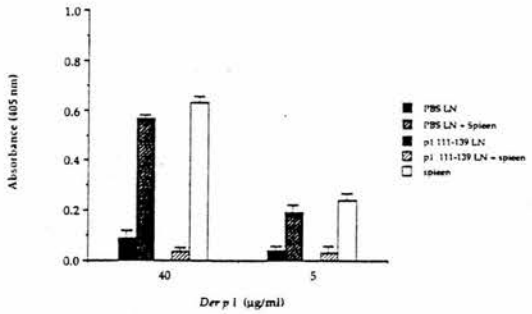

Figure 2. Inhibition of antibody production in vitro. Spleens were removed from Der p 1 immune mice and the periaortic and inguinal LN were collected from control or tolerized mice who had been immunized with Der p 1/CFA. 4×10^5 spleen or LN cells were cultured alone or 2×10^5 spleen cells were mixed with equal numbers of LN cells from either control or tolerized mice. All cells were cultured for 7 d at 37°C and supernatants were assayed for Der p 1-specific Abs using an ELISA assay. Data show the mean response from five mice per group and is presented as ELISA OD units at 405 nm.

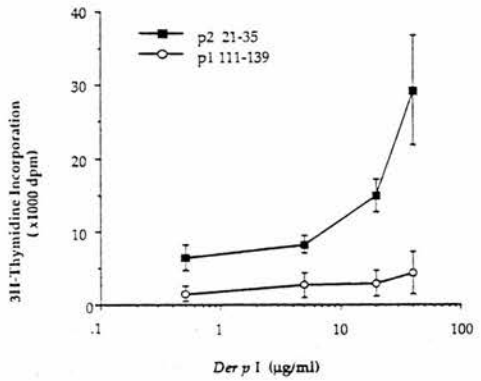

Figure 3. Peptide therapy inhibits ongoing immune responses to the Der p 1 allergen. Mice were treated on five consecutive days with p1 111–139 (○) or a control peptide (■) 10 d after sensitization with Der p 1. 1 wk after the completion of therapy mice were reimmunized with Der p 1/IFA and LN cells were collected 7 d later. The data shows the mean IL-2 response of individual mice ± SD. The number of animals per group used in each experiment were control (*p2 21–35*) $n = 5$, and tolerized (*p1 111–139*) $n = 10$. This experiment has been repeated with over 20 animals with equivalent results.

tants of LN cells from mice tolerized with p1 111–139. When LN cells from PBS-treated mice were mixed with immune spleen cells, Ab production was unaffected (Fig. 2). However, addition of LN cells from p1 111–139-treated mice to spleen cell cultures completely inhibited antibody production in vitro (Fig. 2). The mechanisms which lead to inhibition of antibody synthesis have all the hallmarks of being classical suppression. That is, not only do tolerized T cells fail to provide cognate help to primed B cells in vitro, but the tolerized cells also have the capacity to suppress the effector function of primed $CD4^+$ Th cells in the spleen.

Since allergy to HDM reflects a chronic disease, we wished to examine whether it were possible to inhibit an on-going T cell response using intranasal peptide therapy (16). Mice were immunized with Der p 1 in CFA and 10 d later they received either 100 μg of p1 111–139 or an irrelevant peptide from the Der p 2 allergen, p2 21–35, intranasally on five consecutive days. After 7 d the mice were reimmunized with Der p 1 in IFA. 1 wk later, LN cells were cultured in vitro with the Der p 1 protein and IL-2 production was measured in supernatants of 24-h cultures. LN cells from control mice treated with the nonimmunogenic peptide p2 21–35 were able to produce IL-2 responses when stimulated with the allergen in vitro (Fig. 3), whereas IL-2 secretion was completely abrogated in LN cells from mice receiving p1 111–139 (Fig. 3). Equivalent results were obtained if mice were immunized with Der p 1/CFA and then 6 mo later received p1 111–139 on five consecutive days and then were rechallenged with Der p 1/IFA 7 d after therapy (Fig. 4). Therefore intranasal peptide therapy will induce tolerance not only in naive mice but also will inhibit the function of T cells previously sensitized to the antigen.

The studies reported here demonstrate that inhalation of peptides in low concentrations will induce peripheral tolerance in mice such that they become profoundly unresponsive to a powerful subcutaneous challenge with the antigen. Peptides containing a dominant T cell epitope were more effective tolerogens than those containing only minor epitopes, since tolerance could be induced in naive mice with only one exposure to p1 111–139, whereas a minimum of three doses were required to induce tolerance to a peptide containing a minor epitope (p1 156–168) (data not shown). Furthermore,

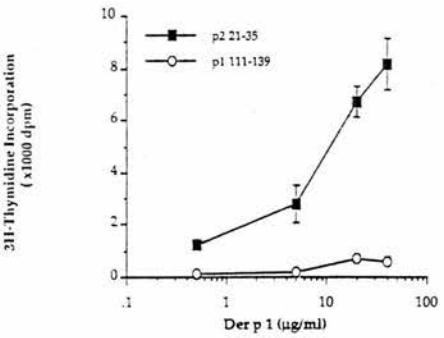

Figure 4. Inhibition of long-term immune responses to Der p 1. C57BL/6J mice were immunized with 100 μg of Der p 1/CFA i.p. and 3 wk later mice were rechallenged with 100 μg of Der p 1/IFA i.p. 6 mo later, half the mice were given 100 μg of p1 111–139 intranasally for 5 d, whereas the other half received p2 21-35 over the same period, and 1 wk later all mice were immunized with 100 μg Der p 1/CFA s.c. at the base of tail and LN cells were cultured 1 wk later. Data shows the mean IL-2 response of five mice per group ± SD.

we found that intranasal peptide therapy does inhibit an ongoing immune response in mice previously sensitized to the HDM allergen. Clearly these results have important implications showing that peptides could be effective immunotherapeutic agents in allergic conditions.

It should be emphasized from these studies that although only one peptide epitope was used for treatment, T cell responses to the other epitopes of the antigen were subsequently downregulated. This spreading of tolerance to encompass all epitopes of a protein antigen shares some similarities to the model of "infectious" tolerance (21). However, we cannot exclude other regulatory mechanisms operating within the lung that may be active in modulating the response antigen-reactive T cells. There is precedence in the literature for a role of $CD8^+$ T suppressor cells in immunological tolerance after inhalation of OVA in rats (13). Therefore, there maybe more than one mechanism operating to control T cell responses to foreign antigens encountered within the nasal mucosa and the lung. Finally, the HDM response in mice provides a promising model for studying further the peptide-induced modulation of allergen-reactive T cells.

We thank Dr. B. A. Askonas for helpful discussions and for carefully reviewing the manuscript.

This work was supported by grants from the Wellcome Trust, the Asthma Foundation of Western Australia, and the National Health and Medical Research Council of Australia. Robyn O'Hehir is the recipient of a Wellcome Senior Research Fellowship in Clinical Sciences.

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Received for publication 20 July 1993.

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International Immunology, Vol. 8, No. 3, pp. 335–342

Regulation of house dust mite responses by intranasally administered peptide: transient activation of CD4$^+$ T cells precedes the development of tolerance *in vivo*

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Keywords: allergy, immunotherapy, T cell cytokines, tolerance

Abstract

We have previously demonstrated that intranasal (i.n.) administration of an immunodominant peptide (p1 111–139) derived from the house dust mite (HDM) allergen Der p 1 inhibits antigen-specific CD4$^+$ T cell responses in H-2^b mice. Here we report that i.n. peptide induced a rapid but transient activation of MHC class II restricted CD4$^+$ T cells that peaked 4 days after peptide treatment and was of similar magnitude to that induced by parenteral immunization with antigen in adjuvant. During the early phase of the response lymph node and splenic T cells secreted a range of lymphokines when re-stimulated *in vitro* with p1 111–139; however, by day 14 IL-2 and IFN-γ secretion by T cells were down-regulated. Mice deficient in CD8$^+$ T cells became tolerant by i.n. treatment with peptide, suggesting that CD8$^+$ T cells are not involved in down-regulating the CD4$^+$ T cell response. Rechallenging mice with a single dose of p1 111–139 21 days after the initial treatment elicited a further transient T cell response, which was subsequently down-regulated over time. Although the i.n. peptide induced a strong transient CD4$^+$ T cell response, only low levels of peptide-specific antibodies were detected either after the initial or subsequent i.n. exposures to p1 111–139. Our findings address the mechanisms underlying peripheral T cell tolerance following i.n. administration of a high dose of immunogenic peptide and have implications for understanding the consequences of peptide immunotherapy.

Introduction

Antigen-specific CD4$^+$ T cells play an important role in allergic sensitization by secreting lymphokines that promote IgE synthesis and support the growth or maturation of effector cells such as eosinophils and mast cells (1). Protein antigens derived from the house dust mite (HDM), *Dermatophagoides pteronyssinus* are a common source of aeroallergens in the environment. ~10% of the population develop allergies to HDM resulting in clinical symptoms including perennial rhinitis, atopic dermatitis or asthma (1). At present desensitization therapy is not successful for most HDM allergic patients and more effective methods of modulating the allergic immune response are required. Recently it has been shown that human HDM-reactive CD4$^+$ T cells can be inactivated *in vitro* by exposing them to supraoptimal concentrations of their cognate peptide (2,3). Furthermore, it is possible to inhibit the function of allergen-reactive T cells *in vivo* by either intranasal (i.n.) or oral administration of a peptide containing the immunodominant T cell epitope derived from the HDM allergen Der p 1 in H-2^b mice (4,5). These results suggest that peptide-mediated immune regulation may be useful in allergen immunotherapy as has been demonstrated in the prevention of experimental autoimmune diseases (6–9).

Administration of antigens via mucosal surfaces is an effective way of inhibiting antigen-specific T cell responses *in vivo*. Oral tolerance to protein antigens results in the inhibition of antigen-specific CD4$^+$ T cell responses *in vitro* by either clonal deletion, clonal anergy or active suppression depending on the dose of antigen administered (10,11).

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Transmitting editor: A. Radbruch

Received 8 August 1995, *accepted* 27 November 1995

Little is known about the mechanisms that regulate immune responses following high-dose antigen treatment through the respiratory tract. In rats continuously exposed to low doses of nebulized protein, $CD4^+$ T cells become primed to the antigen and secrete IL-2 and IL-4 (12). The $CD4^+$ T cell response, however, is then suppressed by the activation of IFN-γ-secreting $CD8^+$ T cells and these suppressor cells abrogate the induction of IgE synthesis in naive recipients upon adoptive transfer (12–14). In this paper we have begun to elucidate the cellular mechanisms which give rise to the development of antigen-specific T cell tolerance following i.n. administration of peptide. We examined the nature of the primary T cell response elicited by high doses of i.n. peptide and investigated the role of $CD8^+$ T cells in the induction of tolerance. We also demonstrate that rechallenging mice with i.n. peptide after 3 weeks after the initial treatment elicits a further transient T cell response to the peptide. These studies have important implications in understanding the response to mucosally delivered peptides in immunotherapy.

Methods

Animals

Inbred female C57BL/6J mice were purchased from Harlan OLAC (Bicester, UK) at 6–8 weeks of age and were kept in isolators. $CD8^{-/-}$ mice were obtained as a fifth generation backcross on the C57BL/6J background and bred under conventional conditions. The mice were kindly provided by Dr Dimitri Kioussis (National Institute of Medical Research, The Ridgeway, Mill Hill, UK).

Antigens

The synthetic peptide p1 111–139 was derived from the Der p 1 sequence and was synthesized using standard f-moc chemistry.

Antibodies

Monoclonal antibodies specific for murine CD4 (YTS.191.2.1) and CD8 (Lyt2, 53.5.) were obtained from the European Tissue Culture Collections. The murine anti-i-A^b mAb (M5/114) was a kind gift from Dr R. Lechler. Cervical lymph node (LN) cells from peptide-treated mice were cultured with mAb supernatants or a control rat anti-mouse IgG at 1/20 and 1/100 dilution in the presence or absence of peptide for 24 h at 37°C. Supernatants were collected and assayed for the presence of lymphokines.

Induction of T cell non-responsiveness by inhalation of peptide

Mice were lightly anaesthetized with ether and the peptide p1 111–139 (100 μg) dissolved in 20 μl of PBS was administered i.n. using a micropipette on three consecutive days. Mice were immunized s.c. at the base of the tail 14 days after the last treatment with 50 μg of Der p 1 emulsified in complete Freund's adjuvant (CFA), (Difco, Detroit, MI) in a volume of 0.2 ml.

Culture medium

LN or spleen cells were cultured in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 2% FCS (Gibco), 50 μM 2-mercaptoethanol (Sigma, St Louis, MO), 2 mM L-glutamine (Sigma) and 20 μg/ml penicillin/streptomycin. CTLL-2 cells were maintained in RPMI 1640 (Gibco) and 10% FCS, while FDC-P1 cells were cultured in DMEM plus 5% FCS.

T cell assays

The cervical LNs were pressed through a stainless steel wire mesh, washed and cultured at 4×10^5 cells in 0.2 ml culture medium in 96-well flat-bottom tissue culture plates (Becton Dickinson Labware, Lincoln Park, NJ) at 37°C. Protein or peptide antigen was added at various concentrations and supernatants were collected at 24 or 48 h and stored at −20°C until assayed.

Lymphokine assays

The CTLL-2 cell line proliferates maximally with IL-2 but only poorly in the presence of IL-4 (15). Test supernatants (50 μl) were added in triplicate to 5×10^3 CTLL-2 cells (50 μl) per well and cultured for 24 h at 37°C. Tritiated methyl thymidine (^{3}[H]TdR; 1μCi, Amersham, Amersham, UK) was added during the last 6 h of culture.

FDC-P1 cells proliferate in response to IL-3 and granulocyte macrophage colony stimulating factor (GM-CSF) (15). FDC-P1 cells (2×10^3 in 50 μl) were cultured with the test supernatants in triplicate wells for 40–48 h and then pulsed with 1 μCi ^{3}[H]TdR for the last 6 h.

CT.4S cells proliferate in response to IL-4, but only poorly to IL-2. Cells (2×10^3) were cultured with test supernatants for 96 h and pulsed with ^{3}[H]TdR for 6 h. The level of radioactivity incorporated by lymphokine dependent cell lines was determined by harvesting cells onto glass fibre filter mats and counted using a Beta Plate Liquid Scintillation counter (Wallac).

IFN-γ was measured using an ELISA assay. Briefly, rat anti-mouse IFN-γ mAb (R4–6A2; PharMingen) was coated onto Immulon-2 plates overnight and washed with PBS containing 0.05% Tween 20 (Sigma). Plates were blocked with 10% BSA dissolved in PBS. After washing, culture supernatants were added to the plates and incubated for 2 h at room temperature. Plates were washed and the presence of bound IFN-γ detected by biotinylated anti-mouse IFN-γ (XMG1.2, PharMingen). Plates were developed using streptavidin-alkaline phosphatase (Sigma) with 3,3′-5,5′-tetramethyl benzidine (Sigma) as the substrate. Plates were read at 405 nm.

Antibody measurements

The p1 111–139 peptide was coated onto Immulon-4 microtitre plates at 10 μg/ml overnight at 4°C. Plates were blocked with PBS containing 10% BSA. After washing, serum dilutions were added to the plate and incubated for 2 h at room temperature. Plates were washed and the presence of bound antibody was detected isotype-specific biotinylated antibodies (Southern Biotechnology Associates). Plates were washed and developed using streptavidin–alkaline peroxidase (Sigma) with 3,3′-5,5′-tetramethyl benzidine (Sigma) as the substrate. Results expressed as OD absorbance units at 405 nm.

Results

T cells undergo transient activation during the induction phase of non-responsiveness

In order to investigate the effect of i.n. peptide on the effector function of antigen-specific T cells *in vivo*, C57BL/6J mice were treated i.n. on three consecutive days with 100 μg of p1 111–139. This regime has previously been shown to be effective in inducing peripheral tolerance to Der p 1 in naive mice. The response of T cells in the draining cervical LN and spleen of i.n. treated mice was examined at various times over a 28 day period and their capacity to secrete lymphokines following antigenic challenge *in vitro* was determined. When T cells are re-stimulated with peptide *in vitro* on day 2 they secrete high levels of IL-3/GM-CSF but only low levels of IFN-γ and IL-2 (Fig. 1). By day 4, p1 111–139-specific T cells in the LN and spleen display their highest level of secretion of IL-2, IL-3/GM-CSF and IFN-γ (Fig. 1) with little or no IL-4 (not shown). By day 8 T cell responses in the cervical LN and spleen had begun to decline, and on day 14 and day 28 peptide-specific T cells secreted only low levels of IL-3/GM-CSF and no IL-2 or IFN-γ when stimulated *in vitro* (Fig. 1). During the first 4 days after peptide inhalation there was a rapid increase in the cellularity of the cervical LN that declined over time (Fig. 1d). Spleen cell numbers, however, did not change significantly over the same period. Thus it would appear that by day 14 antigen-specific T cells were anergic as defined by their inability to secrete IL-2 when stimulated *in vitro*.

Identification of the phenotype of the cell responding to peptide

It was important to identify the phenotype of the T cells which responded to the i.n. peptide. Mice were treated i.n. with 100 μg of p1 111–139 on three consecutive days and 4 days later the cervical LN were cultured with 10 μg/ml p1 111–139 either in the presence or absence of mAbs against CD4, CD8, class II MHC or a control antibody (rat anti-IgG). Cells treated with the control antibody responded strongly to the peptide *in vitro* secreting IL-2, IL-3/GM-CSF (Fig. 2) and IFN-γ (not shown). Treating cells with a 1/20 dilution of anti-CD4 mAb inhibited lymphokine secretion in response to the peptide, while anti-CD8 antibody had no effect (Fig. 2). Treating cells with an antibody to class II MHC (anti-i-A^b) also abrogated the secretion of lymphokines in response to peptide stimulation *in vitro* (Fig. 2). These results indicate that the cells responding

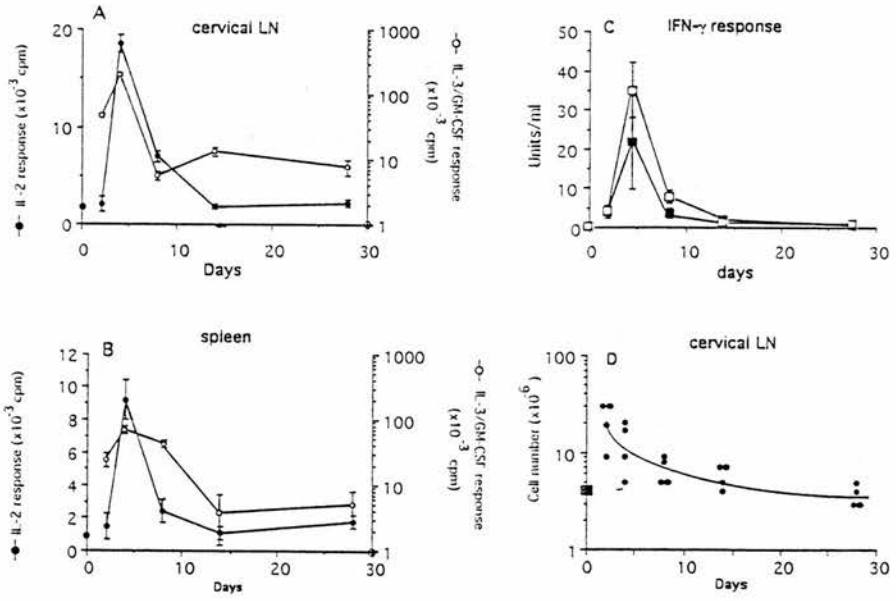

Fig. 1. Lymphokine production by T cells following i.n. administration of peptide. Mice were treated with 100 μg p1 111–139 on three consecutive days, and (A) the cervical LN and (B) spleen were cultured *in vitro* on days 2, 4, 8, 14 and 28 post-i.n. treatment and stimulated with the peptide. (A and B) Supernatants were collected and assayed for the presence of IL-3/GM-CSF (○) and IL-2 (●). (C) Cervical LN (□) and spleen (■) cells were cultured *in vitro* with p1 111–139 and the secretion of IFN-γ was detected in 48 h supernatants. The data show the mean response of five mice per time point ± SD. The data presented is representative of three separate experiments. LN or spleen cells in these experiments were cultured in the presence of varying concentrations of p1 111–139, but only results for 10 μg/ml are shown since this is the dose of peptide that gives optimal T cell activation *in vitro*. Responses from naive T cells stimulated with p1 111–139 (10 μg/ml) on day 0 are usually <1000 c.p.m. for IL-2 and <800 c.p.m. for IL-3/GM-CSF. (D) Data show the average cell yields from the cervical LN and is from one representative experiment.

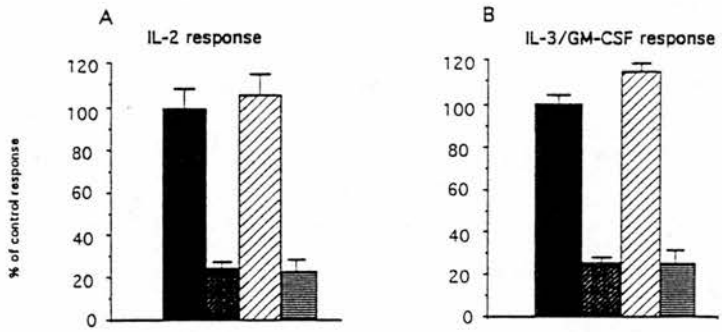

Fig. 2. CD4⁺ T cells respond to the i.n. peptide. Mice were treated with p1 111–139 i.n. and 4 days later the cervical LN were cultured *in vitro* with the peptide at 10 μg/ml. Cells were treated with a control rat anti-IgG (■) or mAbs against either CD4 (▨), CD8 (▨) or I-A^b (▤) (see Methods). Supernatants were then assayed for the presence of IL-3/GM-CSF or IL-2. Results are expressed as mean response of five mice ± SD.

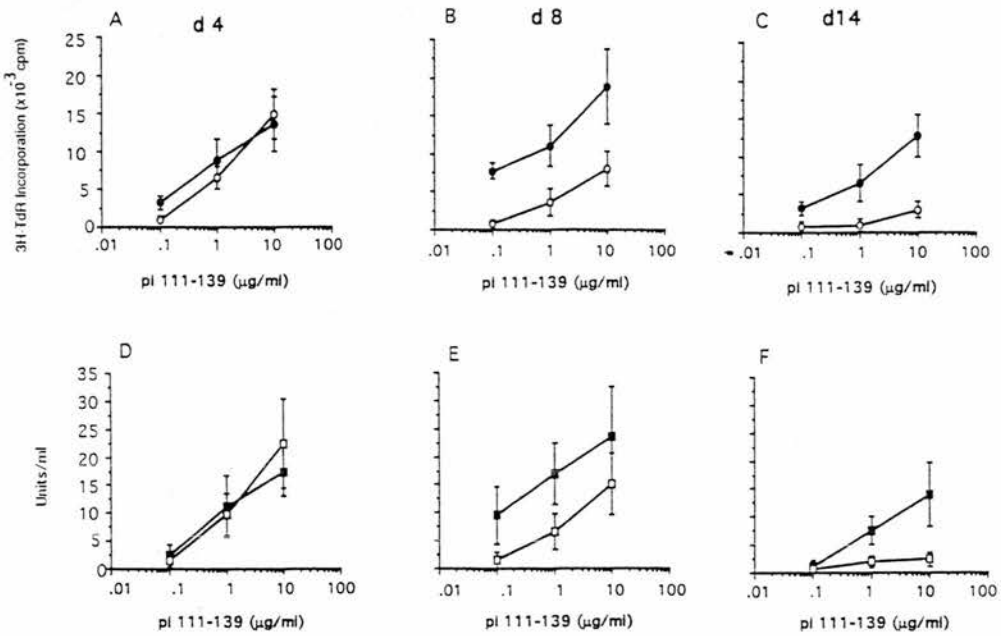

Fig. 3. The induction of tolerance leads to an equivalent level of T cell activation. Mice were treated with 3×100 μg of p1 111–139 i.n. or 50 μg Der p 1/CFA s.c. at the scruff of the neck and the cervical LN (■) and spleen (□) cell responses were examined *in vitro* 4, 8 and 14 days after the final challenge. Data show the mean (A–C) IL-2 and (D–F) IFN-γ response from five mice per time point ± SD.

to high-dose HDM peptide are class II MHC restricted and express CD4 co-receptor.

Comparison of antigen-specific T cell responses following an activating or tolerizing challenge with antigen

Mice were treated i.n. with 3×100 μg of p1 111–139 or were immunized at the scruff of the neck with 50 μg Der p 1/CFA. T cell responses were measured in the cervical LN on days 4, 8 and 14 after both forms of challenge, and production of IL-2 and IFN-γ was examined. On day 4 there was little difference in the T cell responses of mice receiving the tolerizing or activating challenges with antigen. T cells from both groups secreted equivalent levels of IL-2 and IFN-γ (Fig. 3). However, by day 8 T cells from the peptide-treated

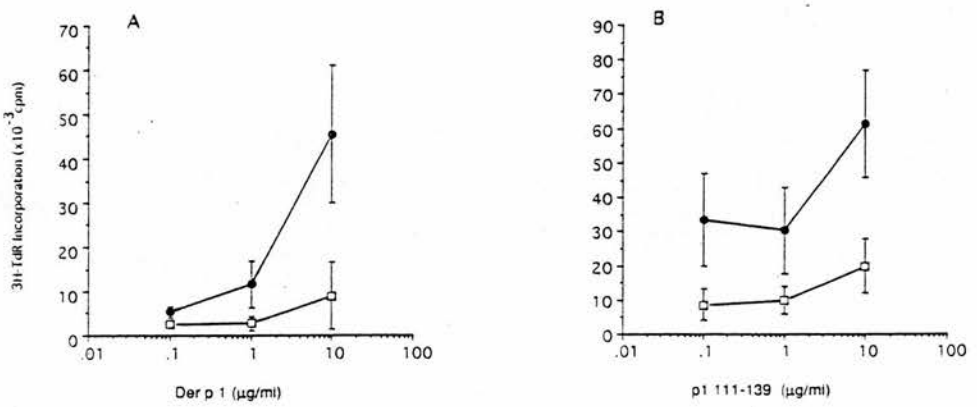

Fig. 4. CD8⁺ T cells do not play a role in tolerance to i.n peptide. CD8$^{-/-}$ mice were treated with either PBS or p1 111–139 i.n. in a typical tolerance experiment. At 10 days after parenteral challenge with Der p 1/CFA LN cells were cultured *in vitro* in the presence or (A) Der p 1 or (B) p1 111–139. Data show the mean IL-2 response from five mice per group ± SD.

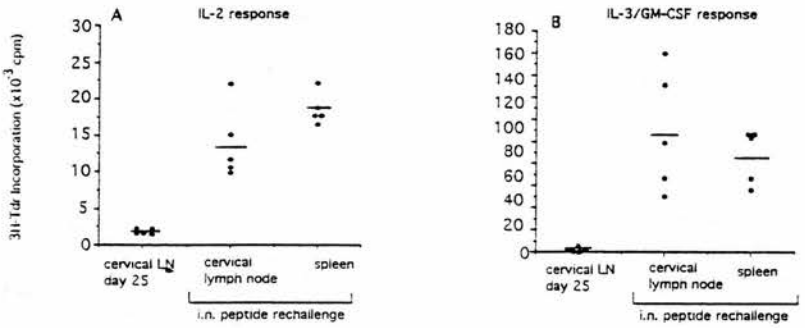

Fig. 5. Antigen-specific T cells display a further transient response after rechallenge with i.n. p1 111–139. Mice were treated with 100 μg p1 111–139 on three consecutive days and 21 days later mice were given a single 100 μg dose of p1 111–139 i.n., while other mice were left untreated. Four days post-challenge, the cervical LN and spleen cells were cultured *in vitro* with p1 111–139 and the 24 h supernatants were assayed for the presence of (A) IL-2 and (B) IL-3/GM-CSF. Data shows the mean response of five mice per group ± SD. The data are representative of three separate experiments.

mice had begun to down-regulate their cytokine secretion, and this was most noticeable by day 14 (Fig. 3).

CD8⁺ T cells do not play a role in the maintenance of peripheral tolerance to inhaled peptide

To examine whether regulatory CD8⁺ T cells play a role in inhibiting the function of antigen-specific CD4⁺ T cells following i.n. peptide treatment we used mice deficient in CD8⁺ T cells. The CD8$^{-/-}$ mice were treated with PBS or with p1 111–139 i.n. using the standard tolerance protocol. Following Der p 1 immunization, LN cells from PBS treated animals were still responsive and could secrete IL-2 when stimulated *in vitro* with either Der p 1 or the dominant peptide (Fig. 4). CD8$^{-/-}$ mice that had been pretreated with p1 111–139 i.n. had down-regulated antigen-specific T cell responses to both the protein and the peptide, typical of wild-type mice (Fig. 4).

Intranasal peptide challenge in vivo after anergy induction results in further transient antigen-specific IL-2 production

Twenty-one days after i.n. peptide, antigen-specific T cells in the regional LN and spleen fail to secrete IL-2 when stimulated *in vitro* with p1 111–139. Thus we wished to examine if peptide-specific T cells would remain anergic after a further i.n. challenge with p1 111–139. Four days after the *in vivo* rechallenge, T cells from the cervical LN and spleen secreted IL-2 and IL-3/GM-CSF in an antigen-specific manner when stimulated *in vitro* with peptide (Fig. 5). In comparison, T cells from unchallenged mice failed to secrete IL-2 and only low

levels of IL-3/GM-CSF (Fig. 5). However, the secretion of IL-2 by peptide-specific T cells was only transient, and by day 14, T cells had again down-regulated IL-2 production. It should be noted that despite the transient release of IL-2 from T cells upon rechallenge with peptide *in vivo* mice remained unresponsive to a challenge with Der p 1/CFA.

We were interested in determining if the same effect would be seen after parenteral challenge with Der p 1. Mice were treated with PBS or p1 111–139 and challenged 2 weeks later with Der p 1/CFA s.c. at the base of tail. LN cells were collected either on day 4 or 10 post-challenge and examined for their capacity to secrete IL-2 following *in vitro* stimulation with p1 111–139. T cells from control mice could secrete IL-2 at high levels on both days 4 and 10 post-challenge (Fig. 6), whereas on the other hand T cells from peptide-treated mice failed to secrete IL-2 at either time point (Fig. 6).

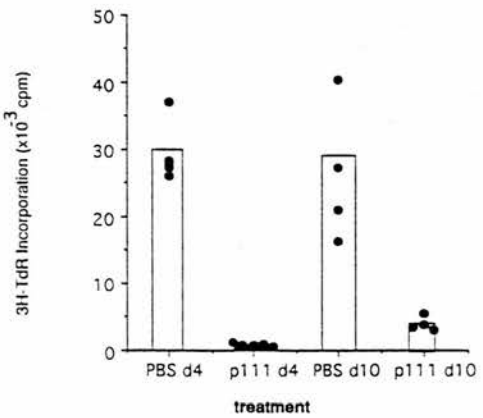

Fig. 6. Intranasally treated mice remain unresponsive following challenge with HDM protein. Mice were treated with PBS or p1 111–139 and immunized 2 weeks later with Der p 1/CFA. LN cells were tested for their capacity to secrete IL-2 on days 4 and 10 post-challenge. The results show the 24 h IL-2 response from T cells of individual mice.

Peptide inhalation does not elicit a significant antibody response

Since inhalation of peptide induced a vigorous T cell response we wanted to determine if there were any serum antibodies produced to the peptide. Mice were treated with 3×100 µg p1 111–139 i.n. and bled on day 14 after the last treatment (Group 1, Fig. 7). The level of peptide-specific antibodies in the serum was low and predominantly of the IgM isotype with a small amount of IgG2a (Fig. 7). Unlike the T cell response we did not observe any significant rise either in the level of peptide-specific antibodies or a switch to the IgG isotype in mice who were rechallenged twice with p1 111–139 i.n. at fortnightly intervals (Group 2, Fig. 7).

Discussion

We report here that i.n. administration of a high dose of HDM-derived peptide elicits a strong but transient activation of CD4⁻ T cells that eventually gives rise to a state of antigen-specific T cell non-responsiveness. Responses to the i.n. peptide spread systemically into various lymphoid tissues within 48 h, but are strongest in the draining cervical LN. The peptide elicited a vigorous response *in vivo* as indicated by a 3- to 4-fold increase in the cellularity of the cervical LN over the first 4 days. The T cell responses peaked on day 4 after the completion of treatment, at which time the cells were capable of secreting IL-2, IL-3/GM-CSF and IFN-γ but with little or no IL-4 when stimulated with p1 111–139 *in vitro*. By 2 weeks, peptide-specific T cells had down-regulated IL-2 and IFN-γ secretion but there was residual antigen-specific secretion of IL-3/GM-CSF responses even on day 28. In addition we noticed that the activation of T cells following peptide inhalation appeared to be of the same magnitude as that elicited by a conventional immunization with Der p 1 in CFA.

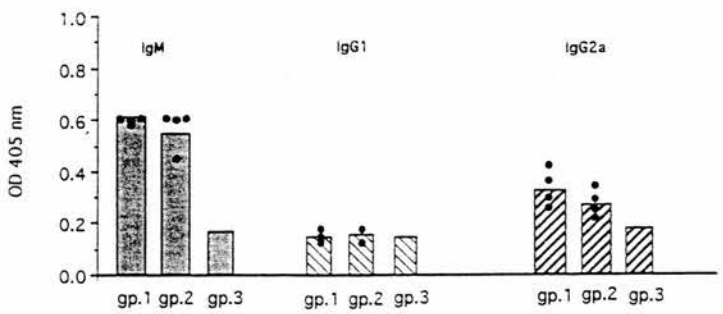

Fig. 7. Intranasally administered peptide induces minimal antibody responses *in vivo*. Group 1 mice were treated with 100 µg p1 111–139 i.n. on three consecutive days and 14 days later were bled. Group 2 mice received the same initial treatment but also were rechallenged with p1 111–139 on days 17 and 31, and were bled on day 45. The serum from individual mice of both groups was analysed for the presence of IgM, IgG1 and IgG2a peptide-specific antibodies by ELISA. Group 3 refers to control responses obtained with normal mouse serum.

The failure of T cells to secrete IL-2 following antigenic stimulation has been used as a definition for clonal anergy (16). However, from our studies it would appear that the development of anergy *in vivo* occurring as a consequence of i.n. peptide is mediated by a different mechanism to that proposed for anergy *in vitro* (17,18). After peptide i.n. $CD4^+$ T cells still secrete IL-2 , and therefore, must have received costimulation from antigen-presenting cells (APCs). Nevertheless, the signals which mucosal APCs deliver to the naive T cells must in some way prime these cells to become non-responsive (anergic). Studies to date have suggested that mucosal APCs do not have an intrinsic tolerogenic capacity (19). Whether or not certain populations of APCs are able to deliver inhibitory signals that override the known positive co-stimulatory signals such as those mediated by the B7 family of molecules is not known.

Our findings are consistent with previous studies which have also demonstrated that $CD4^+$ or $CD8^+$ T cells responding to various tolerogens undergo a state of transient activation prior to the development of non-responsiveness (20–24). However, we have obtained no direct evidence for a shift in cytokine production by $CD4^+$ T cells over time following the induction of tolerance to i.n. peptide. This is in contrast to recent findings which show that the development of peripheral $CD4^+$ T cell tolerance following immunization of mice with ovalbumin in incomplete Freund's adjuvant (25). Others suggest a role of $CD8^+$ T_s cells in the regulation of IgE responses in mice and rats exposed to nebulized antigen (13,14,26). However, we failed to demonstrate that $CD8^+$ T cells regulate either the induction or maintenance of tolerance to peptide administered i.n., similar to that described for the induction of oral tolerance (27). There is a possibility that $CD8^+$ T_s cells may function when low doses of antigen are encountered by the immune system, such as in the maintenance of oral tolerance induced by feeding low doses of protein antigen (10). It should be noted that we have only examined responses to high doses of peptide given i.n., which may occur via distinct mechanisms.

Two weeks after peptide treatment, T cells in the draining cervical LN failed to secrete IL-2 when challenged *in vitro*. However, if mice were challenged 21 days after their initial treatment with a single 100 μg dose of peptide i.n., then within 4 days of the challenge, antigen-specific T cells capable of secreting IL-2 were detected in the cervical LN and spleen. The capacity of these T cells to secrete IL-2 was only transient and was down-regulated by 2 weeks. It would appear that IL-2 production on peptide rechallenge *in vivo* did not abrogate peripheral tolerance to the HDM protein, since these same animals were still unresponsive to a parenteral challenge with Der p 1/CFA if given 14 days after the i.n. rechallenge. Furthermore, if mice are continuously rechallenged with peptide i.n. once every 2 weeks then by day 4 after the last rechallenge the presence of IL-2 secreting cells in the cervical LN and spleen is observed. Similar responses have also been shown to occur with periodic feeding of high doses of antigen to mice (28) and with tolerance to the superantigen staphylococcal enterotoxin B (SEB) (29). Following the induction of peripheral tolerance to SEB $V_\beta 8^+$ $CD4^+$ and $CD8^+$ T cells appear to be anergic when stimulated with antigen *in vitro*. However, on rechallenge with SEB *in vivo* $V_\beta 8^+$ TCR^+ T cells regain the capacity to secrete IL-2 but only $CD8^+$ T cells were shown to undergo clonal expansion (29). It is unclear at this stage why the transient IL-2 response is not observed following parenteral challenge with Der p 1 in peptide treated mice. This may be due to differences in epitope concentration or may involve different APCs. Despite the strong transient $CD4^+$ T cell response that occurs to the i.n. peptide these cells fail to provide effector help to B cells to induce Ig class switching. Although some IgM peptide-specific antibodies are present, no IgG antibodies can be detected in the serum of mice. Our findings suggest that the transient IL-2 response observed after peptide rechallenge is from naive T cells that have not yet been tolerized to the peptide. What is clear from these experiments is that the development of peripheral tolerance to i.n. administered, or fed, antigens is not complete after the first encounter with antigen, but instead the immune system continuously responds to these antigens in a negative way that acts to preserve tolerance.

It is possible that the development of mucosal tolerance involves more than one mechanism in order to control responses to common dietary or inhaled antigens. For example, following high-dose antigen treatment some T cells may undergo apoptosis as a result of antigen recognition (11,30), a subset of cells may escape death but become anergic (31). In addition, negative responses mediated by regulatory T_s cells may be superimposed on these effects (14,26). Therefore, different overlapping mechanisms of immune regulation at mucosal surfaces may provide for an efficient control over immune responses to normal environmental antigens.

Our results have important implications for the use of peptides in immunotherapy. Currently a clinical trial is in progress investigating the efficacy of synthetic peptides derived from the Fel day 1 allergen to ameliorate allergic symptoms in cat allergic individuals (32). Our results are likely to provide an insight into the mechanisms of how mucosally delivered peptides induce peripheral tolerance in $CD4^+$ T cells *in vivo*. In addition we have demonstrated that the immune system continuously responds to the peptide tolerogen on periodic challenge *in vivo*. This is an important observation since patients undergoing peptide immunotherapy are expected to receive peptide injections every 3–4 weeks over an extended period of time. Clearly information is required to better understand the cellular and molecular basis for tolerance induction by peptides delivered through mucosal surfaces. This will require examination of the nature of mucosal APCs and the nature of the signals delivered to the T cells.

Acknowledgements

This work was supported by grants from the Wellcome Trust and the Medical Research Council, UK.

Abbreviations

APC	antigen-presenting cells
CFA	complete Freund's adjuvant

GM-CSF	granulocyte macrophage colony stimulating factor
HDM	house dust mite
i.n.	intranasal
LN	lymph node
SEB	staphylococcal enterotoxin B

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International Immunology, Vol. 9, No. 8, pp. 1165–1173

Characterization of the specificity and duration of T cell tolerance to intranasally administered peptides in mice: a role for intramolecular epitope suppression

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Keywords: antigen presentation, delayed-type hypersensitivity, epitopes, house dust mite, IgE, intramolecular epitope suppression, T cell tolerance

Abstract

Mucosal administration of antigens in experimental animals leads to the induction of peripheral T cell tolerance. We have previously reported that in H-2^b mice, intranasal (i.n.) or oral administration of a peptide containing the immunodominant T cell epitope will down-regulate the function of CD4$^+$ T cells reactive with Der p 1, a major target antigen in both B and T cell responses to house dust mite. In the present study we have investigated the tolerogenicity of peptides containing both dominant and subdominant determinants when given i.n. to naive mice. Induction of tolerance by the nasally administered immunodominant peptide leads to a diminution in all T cell-derived cytokines and modulation of delayed-type hypersensitivity responses, but IgE production did not seem to be affected, furthermore the induction of T cell tolerance was stable, lasting beyond 6 months. We have also examined the specificity of intramolecular epitope suppression which is a feature of mucosal tolerance induced by nasally administered peptides and demonstrate that regulatory CD4$^+$-T cells may exert their suppressive effect by linked recognition of epitopes on the same or neighbouring antigen-presenting cells.

Introduction

Soluble protein antigens encountered through the respiratory or gastrointestinal tracts do not elicit strong systemic immune responses but induce a state of antigen-specific unresponsiveness which is commonly referred to as mucosal tolerance. It is characterized by a diminution of T cell effector responses and consequently antibody synthesis. As regards the cellular mechanisms responsible for the development of tolerance, it appears that feeding high doses of antigen can lead to the deletion of antigen-specific T cells (1), anergy (2–4) or a shift in the cytokine production of T cells (5). Similarly, tolerance to inhaled antigens can arise as a result of anergy or immune deviation (6,7).

Early studies on mucosal tolerance were restricted to analysis of cellular responses to whole protein antigens, but it now appears that immunogenic peptides which contain T cell epitopes can also act as potent tolerogens *in vivo* (8–12). Peptides derived from self antigens delivered in tolerogenic form through mucosal surfaces can prevent the induction of, or reduce the severity of, clinical symptoms associated with various autoimmune diseases (12–14). Furthermore, we have previously reported that the intranasal (i.n.) or oral administration of a single immunodominant peptide derived from the house dust mite (HDM) protein Der p 1, when given prior to immunization with the whole protein, can induce peripheral tolerance in both naive (15,16) or sensitized mice (15). The non-responsiveness is characterized by a reduction in IL-2 production by both lymph node and splenic T cells, and their failure to provide cognate help for antibody production (15).

A feature of peptide-induced mucosal tolerance is that treatment with a single immunogenic peptide can abrogate T cell responses to all the epitopes on an antigen

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Transmitting editor: A. Radbruch

Received 23 September 1996, *accepted* 29 April 1997

(13–16). Such intramolecular, and presumably intermolecular (or bystander), epitope suppression seems to be effective in inhibiting the onset of clinical symptoms in different autoimmune disease models (13,14,17), where immunological responses to multideterminant self antigens are typically involved. These studies and others (18,19) have suggested that peripheral T cell tolerance can occur through linked recognition of tolerogenic antigen which helps to block an antigen-specific immune response. In the present study we have investigated further the specificity and duration of the T cell tolerance to peptides delivered i.n. to mice.

Methods

Animals

Inbred C57BL/6J mice were purchased from Harlan Olac (Bicester, UK) or Murdoch Univeristy (Western Australia) at 6–8 weeks of age and were kept in isolators. All experiments were performed in accordance to the animal ethics regulations of the Home Office in the UK and the NH&MRC guidelines in Australia.

Antigens

The HDM allergen Der p 1 was affinity purified from spent mite medium (16). Synthetic peptides p81–102, p111–139 and p197–212 derived from the Der p 1 sequence were synthesized using standard f-moc chemistry.

Induction of tolerance by i.n. administered peptides

Mice were lightly anaesthetized under ether and peptides dissolved in PBS were administered i.n. in a total volume of 20 µl using a micropipette on three consecutive days. Mice were immunized s.c. at the base of the tail 14 days after the last treatment with 50 µg of Der p 1 emulsified in complete Freund's adjuvant (CFA; Difco, Detroit, MI) in a volume of 0.2 ml. Ten days later the para-aortic and inguinal lymph nodes were collected and cell suspensions made. To examine bystander suppression to an unrelated antigen, peptide-treated or control mice were co-immunized with 50 µg Der p 1 and ovalbumin (OVA) in CFA.

Culture medium

Lymph node (LN) or spleen cells were cultured in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 2% FCS (Gibco), 50 µM 2-mercaptoethanol (Sigma, St Louis, MO), 2 mM L-glutamine (Sigma) and 20 µg/ml penicillin/streptomycin. CTLL-2 and MV1-Lu cells were maintained in RPMI 1640 and 10% FCS, and FDC-P1 cells were cultured in DMEM (Gibco) plus 5% FCS.

T cell assays

LN cells were pressed through a stainless steel wire mesh, washed and cultured at 4×10^5 cells in a volume of 0.2 ml in culture medium in a 96-well flat-bottom tissue culture plate (Nunc, Copenhagen, Denmark). Peptide antigen was added at various concentrations and the cells were incubated at 37°C for 24 or 48 h. Supernatants were collected and stored at –20°C until required for the assay.

Lymphokine assays

Cytokine measurements were made with the use of either bioassays [IL-2, IL-4 and transforming growth factor (TGF)-β1] or ELISA (IFN-γ and IL-5). All cytokines were measured in culture supernatants taken at 24 h intervals over a 96 h period. IL-2 and IL-3 were usually measured at 24 h, IL-5, IFN-γ and TGF-β at 48 h, and IL-4 at 96 h.

The CTLL-2 cell line proliferates maximally with IL-2 but only poorly in the presence of IL-4 (20). Test supernatants (50 µl volumes) were added to 5×10^3 CTLL-2 cells (50 µl) per well and cultured for 24 h at 37°C and pulsed for 6 h with [^{3}H]thymidine (1 µCi; Amersham, Amersham, UK).

FDC-P1 cells proliferate maximally to IL-3 and granulocyte macrophage colony stimulating factor but poorly to IFN-γ. Cells (2×10^3) were cultured with test supernatants for 24 h for FDC-P1 cells and 96 h for CT.4S cells and pulsed with [^{3}H]thymidine.

CT.4S cells proliferate in response to IL-4, but only poorly to IL-2 (20). Cells (2×10^3) were cultured with test supernatants for 24 h for FDC-P1 cells and 96 h for CT.4S cells and pulsed with [^{3}H]thymidine.

IFN-γ was measured using an ELISA assay. Briefly, rat anti-mouse IFN-γ mAb (R4-6A2; PharMingen, San Diego, CA) was coated onto Immulon-2 plates overnight and washed with PBS containing 0.05% Tween 20 (Sigma). Plates were blocked with 10% BSA dissolved in PBS. After washing, culture supernatants were added to the plates and incubated for 2 h at room temperature. Plates were washed and the presence of bound IFN-γ detected by biotinylated anti-mouse IFN-γ (XMG1.2; PharMingen). Plates were developed using streptavidin–alkaline phosphatase (Sigma) with 3,3′-5,5′-tetratmethybenzidine (Sigma) as the substrate and plates were read at 405 nm. The concentration of IFN-γ was measured from a standard curve using recombinant IFN-γ (PharMingen).

IL-5 was measured in 48 h culture supernatants by ELISA using a commercially available kit (Endogen IL-5) and was performed following the manufacturer's directions. Standard recombinant IL-5 preparations were included in each assays.

TGF-β1

The growth of the mink lung epithelial cell line MV-1Lu is arrested in the presence of TGF-β1. Test supernatants (50 µl volumes) were added to 2×10^3 MV-1Lu cells (50 µl) per well and cultured for 24 h at 37°C and pulsed for 6 h with [^{3}H]thymidine. The concentration of TGF-β1 in supernatants was determined from a standard curve using purified TGF-β1.

Skin testing for delayed-type hypersensitivity (DTH) responses

Mice received either PBS or p111–139 nasally on three consecutive days and 9 days later mice were immunized with 100 µg Der p 1/CFA. After 7 days mice were injected intradermally with 10 µg Der p 1 in 10 µl PBS. Ear swelling was measured after 24 h and the increments were obtained by subtracting values of the test groups from those control mice who received either PBS or p111–139 alone.

Passive cutaneous anaphylaxis (PCA) assay

Mice were treated nasally with PBS or a tolerogenic dose of peptide 111–139 and then immunized 9 days later with Der

p 1/CFA. After 21 days mice were bled and the serum was serially diluted in doubling dilutions in PBS. Serum was injected intradermally into the back of an adult male Sprague-Dawley rat. After 24 h the rat was anaethetized and injected with 10 μg of Der p 1 in saline containing Evans blue. Serum from Der p 1 hyperimmunized mice was used as a positive control. A positive result was scored at the serum dilution which gave a wheal of 10 mm or larger.

Statistical analysis

The Student's *t*-test and the Mann–Whitney *U*-test was used to analyse the statistical significance of the experimental data where appropriate.

Results

Comparison of peripheral non-responsiveness in H-2^b mice induced by i.n. administration of peptides containing immunodominant or minor T cell epitopes of Der p 1

We have previously described the dominant or subdominant T cell epitopes of the HDM allergen Der p 1 which are recognized following immunization of H-2^b mice with affinity-purified protein emulsified in CFA (16). Peak responses are observed to peptide 110–131, while weaker responses are observed to 81–102, 197–212 and 21–49. The ability of Der p 1-derived peptides containing either the immunodominant (p111–139) or a minor (81–102) epitope to inhibit antigen-specific CD4$^+$ T cell responses when administered nasally was investigated. In all the experiments performed in this report, control mice received PBS i.n., but the specificity of tolerance induction of this model has been previously reported (15). Mice were treated with either PBS or with 100 μg of p111–139 or p81–102 on three consecutive days and immunized with Der p 1/CFA 14 days after the last peptide treatment. The capacity of LN T cells to secrete various cytokines following *in vitro* stimulation with the Der p 1 protein 10 days after the parenteral challenge was examined. All cytokines were measured at various time points from 24 to 96 h in order to determine the optimal secretion and for simplicity cytokines shown in Fig. 1 are only from the peak response. T cells from control mice were still highly responsive to the Der p 1 protein *in vitro* and could secrete IL-2, IL-3, IL-5, IFN-γ but with little or no IL-4, while T cells from mice treated with HDM peptide displayed down-regulated cytokine responses *in vitro* for all the cytokines measured (Fig. 1). In addition, we did not observe any increase in TGF-β1 secretion following tolerance induction to nasally administered peptides (Fig. 1). The level of tolerance achieved with the minor epitope was not as complete as that obtained with the dominant peptide, with the minor epitope only reducing responses to 50–60% of that observed in the control animals (Fig. 1). In all of the remaining experiments we have focused on the secretion of IL-2 by LN T cells since this is the cytokine that is most markedly inhibited in T cells following mucosal tolerance and is a general feature of peripheral T cell tolerance.

Peptide tolerance affects DTH responses but not IgE responses

The above results indicate that T cell responses are markedly reduced following induction of tolerance to nasally administered peptide. To determine if this can be correlated with an *in vivo* response, we compared DTH responses in mice receiving either PBS or p111–139 nasally. Mice were tolerized as usual and after 9 days were immunized with 100 μg Der p 1/CFA. One week later all mice were immunized intradermally in the ear with 10 μg of Der p 1. Specific skin swelling was measured 24 h later. To determine background swelling responses, mice were pretreated with PBS or p111–139 alone and received no Der p 1/CFA injection. The data presented in Fig. 2(A) show that pretreatment with mice with the immunodominant peptide 111–139 could inhibit specific DTH responses to a skin test challenge with Der p 1 *in vivo*, these findings were statistically significant at $P < 0.05$ using both Student's *t*-test and the Mann–Whiney *U*-test.

Serum from the same mice were measured for the presence of specific IgE antibodies against Der p 1 (Fig. 2B). Although peptide tolerized mice displayed markedly reduced CD4$^+$ T cell responses *in vitro* and *in vivo*, antibody responses did not appear to be affected when IgE levels were measured by PCA (Fig. 2B).

Duration of unresponsiveness induced by p111–139

In order to determine if the state of antigen-specific T cell non-responsiveness is long lasting *in vivo*, C57BL/6J mice were treated i.n. with p111–139 or PBS and immunized with Der p 1/CFA 14 days later. Alternatively, mice were treated with 100 μg of p111–139 i.n. on 3 consecutive days and immunized 6 months after the initial treatment and responses examined *in vitro* 10 days after immunization with Der p 1/CFA. T cells from PBS-treated control mice responded well to p111–139 *in vitro* and were capable of secreting IL-2 (Fig. 3), whereas LN cells from peptide-treated animals, i.e. from 2 week or 6 month groups, were still unresponsive to the antigen *in vitro* (Fig. 3).

Characterization of intramolecular epitope suppression

A feature of peripheral tolerance induced by mucosally delivered peptide is that treating mice with a single peptide can inhibit T cell responses to all epitopes on the antigen (13,15), which we define here as intramolecular epitope suppression. We wanted to examine therefore the antigenic requirements that were necessary for this phenomenon to be observed *in vivo*. Mice were treated with either PBS or p111–139 i.n. and 2 weeks later immunized with either 50 μg of Der p 1/CFA, p111–139/CFA or p81–102/CFA. LN T cells from control mice were responsive to each of the immunogens when stimulated *in vitro* (Fig. 4A–C). T cells from mice treated with p111–139 i.n. failed to secrete IL-2 when stimulated *in vitro* with Der p 1 or p111–139 (Fig. 4D and E). However, tolerance could not be demonstrated to the minor peptide *in vitro* if mice pretreated with p111–139 i.n. were immunized with p81–102/CFA (Fig. 4F).

The previous results suggest that the intact protein is required in order to observe intramolecular epitope suppression. The temporal relationship of tolerance to the dominant and minor epitopes was investigated. Mice were treated i.n. with PBS or p111–139 and 14 days later immunized with Der p 1/CFA. In one group *in vitro* T cell responses to the protein and to a minor epitope were examined after the usual 10 day period. T cells from control mice responded well to both Der p 1 and to the minor epitope, p197–212 (Fig. 5A and B).

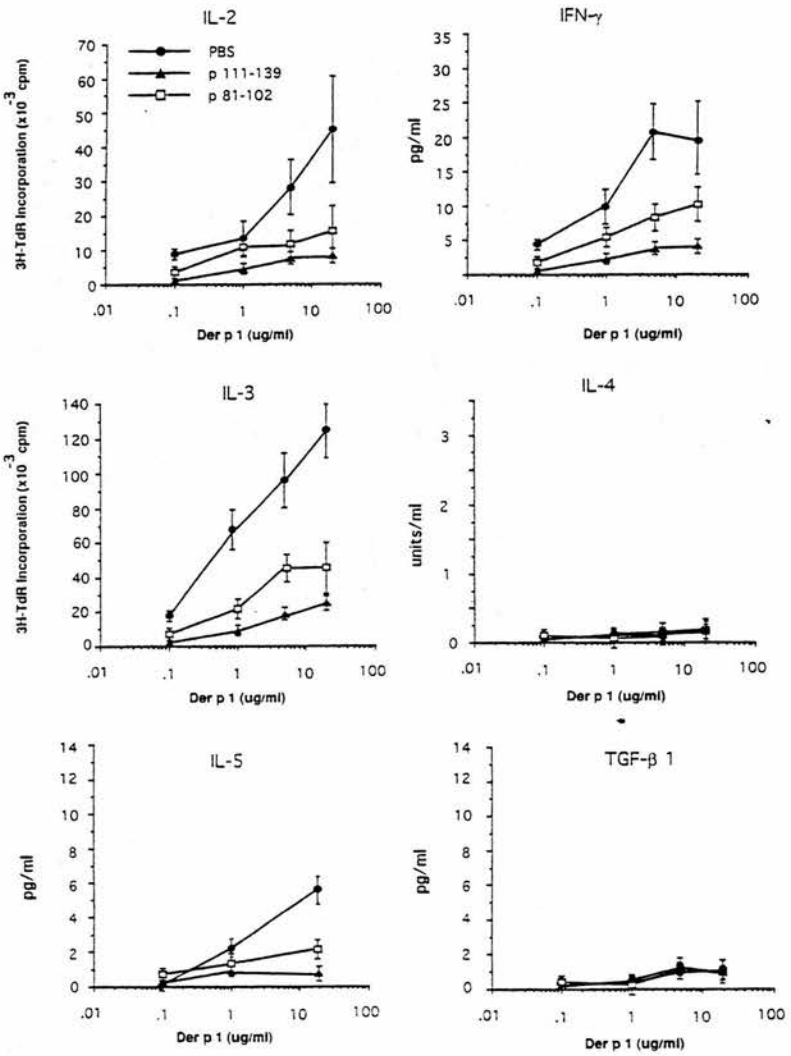

Fig. 1. Modulation in the secretion of T cell-derived cytokines following tolerance induction to nasally administered peptides. Mice were treated i.n. with either PBS (●), p111–139 (▲) or p81–102 (□) and 14 days later challenged with Der p 1/CFA. LN cells from five mice per group were cultured *in vitro* with Der p 1 and cytokines measured after 24 or 48 h. Data shows the mean response ± SD for each of the cytokines. Data have been compiled from at least three experiments.

However, mice that were pretreated with p111–139 showed down-regulated responses to the protein and to p197–212 (Fig. 5A and B). The remaining mice were immunized with 50 μg of 197–212/incomplete Freund's adjuvant (IFA) 3 weeks after the Der p 1 challenge, and after a further 10 days LN cells from both groups were tested for responses to the dominant and minor epitopes. When analysed, T cells from control mice and peptide-tolerated mice displayed equivalent responses to the minor epitope 197–212 (Fig. 5C and D), while T cell responses in both groups to the dominant epitope were still down-regulated.

Bystander suppression

The previous experiments demonstrate that the nasally administered immunodominant peptide can induce regulatory T cells that can block T cell responses to epitopes linked on the

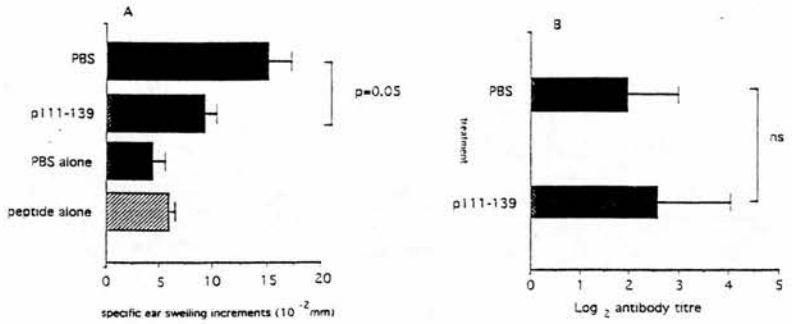

Fig. 2. Tolerance affects T but not B cell responses to Der p 1. (A) Mice were treated with PBS (n = 5) or a tolerogenic dose of p111–139 (n = 6) i.n. and 9 days later mice were immunized with Der p 1/CFA. One week later mice were injected intradermally in the ear with 10 μg Der p 1 in saline. Twenty four hours later skin swelling was measured with a micrometer on the ear injected with antigen and that uninjected. The two values were subtracted from each other to give increments of ear swelling. Control animals received either PBS or p111–139 i.n. only in order to establish background swelling responses. (B) Sera from control or peptide tolerized mice were measured for the presence of allergen-specific IgE by PCA. Serial dilutions of serum were injected intradermally into the back of a Sprague-Dawley male rat and 24 h later the rat was injected with a solution containing 10 μg Der p 1 in Evans Blue. Wheal responses were measured and scored positive responses if they were >10 mm. Data shows the mean antibody dilution to give a positive response.

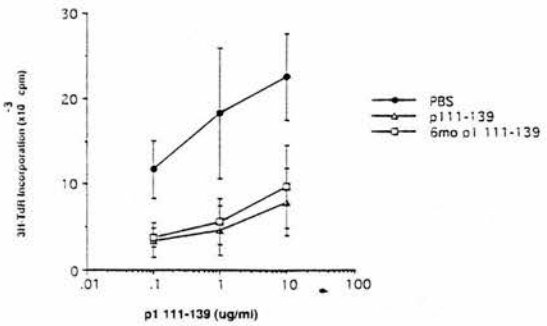

Fig. 3. Peripheral tolerance induced by i.n. peptide persists for long periods *in vivo*. Mice were treated with either PBS (●) or p111–139 and immunized after 2 weeks (△) or received p111–139 i.n. and rested for 6 months before being immunized with Der p 1/CFA (□). Ten days after the immunization LN cells from each group were cultured *in vitro* with the p111–139. Data shows the mean IL-2 response from five mice per group ± SD.

same protein. We wanted to examine if these regulatory T cells may also block immune responses to unrelated antigens *in vivo*. Mice were tolerated with the dominant peptide as usual but on day 14 post-treatment mice were co-immunized with Der p 1 and OVA in CFA. Ten days later LN cells were cultured *in vitro* and responses tested to Der p 1 or OVA separately. As shown in Fig. 6, T cell responses to Der p 1 were clearly abrogated ($P < 0.05$) but although some decrease in T cell responses was observed to the bystander antigen OVA, this was not statistically significant ($P > 0.1$).

Discussion

The delivery of antigens through mucosal surfaces is an efficient way of inducing antigen-specific T cell non-responsiveness, which is referred to as mucosal tolerance. We have previously reported that i.n. administration of the immunodominant peptide (residues 111–139) derived from the Der p 1 allergen of HDM can specifically inhibit CD4$^+$ T cell responses to the whole antigen when administered to naive or sensitized H-2^b mice (15). Several mechanisms have been identified to explain the loss of T cell antigen-reactivity following the induction of mucosal tolerance. Clonal deletion of antigen-reactive T cells has been observed following high-dose feeding of protein antigens to TCR transgenic mice (1), while others have suggested a role for clonal anergy of CD4$^+$ T cells (2,3). In addition there is evidence for active suppression in oral tolerance mediated by TGF-β1 CD8$^+$ T suppressor (T_s) and, more recently, immunoregulatory CD4$^+$ T cells that secrete T_h2-type cytokines (e.g. IL-4, IL-10 and TGF-β1) have been isolated from the Peyer's patch and mesenteric lymph node following feeding with low doses of myelin basic protein (MBP) (5). However, the role for CD8$^+$ T_s cells in oral tolerance is controversial, since several groups have failed to observe a role for such cells in the induction or maintenance of oral tolerance (2,3,21).

Studies on the immune response to nebulized antigens have revealed that allergic sensitization is naturally avoided through a shift in cytokine production (or immune deviation) by specific CD4$^+$ T cells during the course of a primary immune response to the antigen (7). There is evidence that regulatory CD8$^+$ T_s cells become activated following inhalation of protein antigen in experimental animals which can block antigen-specific IgE synthesis *in vivo* (22–25). However, it was recently shown that CD8$^+$ T cells activated by inhaled antigen may play a role in mediating airway hypersensitivity (26). In addition immune deviation was shown to be responsible for the protection of non-obese diabetic mice from autoimmune diabetes following nasal administration of a cocktail of GAD65 peptides (27).

Induction of tolerance to high-dose nasally administered peptide is preceded by a transient response by CD4$^+$ T cells

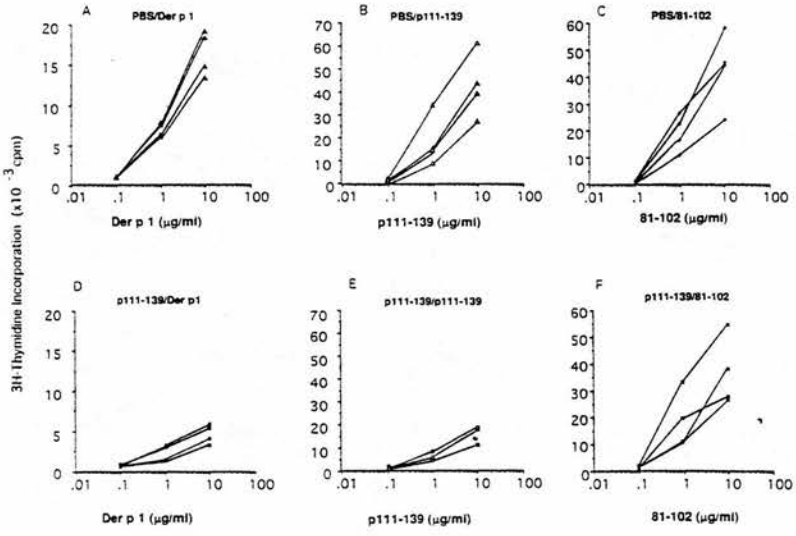

Fig. 4. Molecular requirements for intramolecular epitope suppression. Mice were treated with either PBS (A–C) or p111–139 (D–F), i.n. and 2 weeks later mice were challenged with either Der p 1/CFA (A and D), p111–139/CFA (B and E) or p81–102/CFA (C and F). Data shows the 24 h IL-2 response from LN cultures of individual mice.

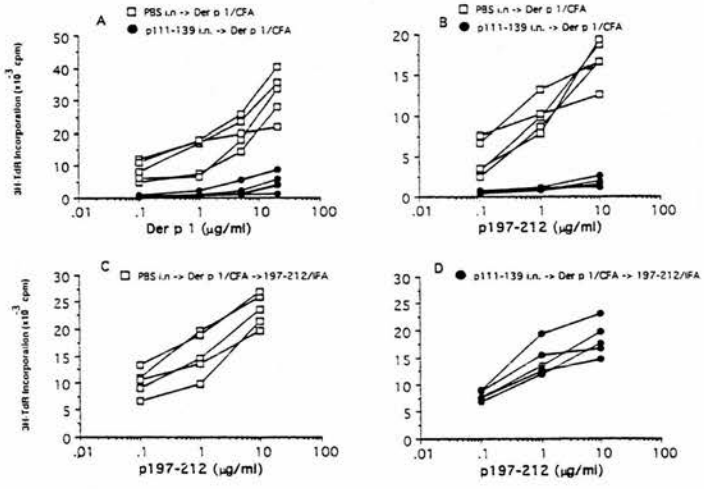

Fig. 5. T cells specific for minor epitopes are not deleted or anergized following i.n. treatment with the immunodominant peptide. Mice were treated i.n. with either PBS (□) or p111–139 (●) and 2 weeks later immunized with Der p 1/CFA. After 10 days half the mice of each group were sacrificed and LN cells tested for responses to (A) Der p 1 and (B) p197–212. The remaining mice were then immunized with p197–212/IFA 3 weeks later and after a further 7 days LN cells were cultured *in vitro* with p197–212 (C and D). Data shows the IL-2 response from individual mice.

(6). We have not identified a role for $CD8^+$ T cells in this model (6) and interestingly do not observe any shift in cytokine production or increased secretion of TGF-β1 by regulatory T cells following i.n. peptide tolerance. This clearly is in contrast to studies on oral tolerance to MBP where TGF-β secretion seems to be an important mechanism for regulating responses of self-reactive $CD4^+$ T cells. In the model studied here, the production of all T-cell-derived cytokines was down-

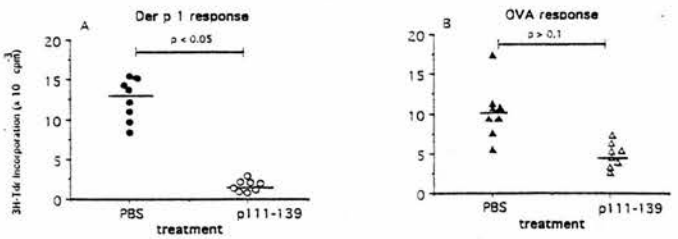

Fig. 6. To examine bystander suppression to a unrelated antigen, Mice were treated with PBS or the dominant peptide i.n. and 14 days later co-immunized with 50 μg Der p 1 + OVA in CFA. Ten days later draining LN cells were cultured *in vitro* with either (A) Der p 1 or (B) OVA. The data show the production of IL-2 in culture supernatants from individual mice stimulated with 20 μg/ml Der p 1 or 400 μg/ml of OVA. Statistical analysis of the data using a Student's *t*-test revealed that the induction of T cell non-responsiveness to Der p 1 is significant at $P < 0.05$ between PBS- and peptide-treated animals, while the lowered OVA responses were not statistically significant between PBS- or p111–139-treated animals ($P > 0.1$).

regulated following tolerance induction with a peptide containing either the dominant or a minor T cell epitope. Although the level of T cell inhibition achieved with the minor T cell epitope was not as profound as that obtained with the immunodominant epitope, our results are in agreement with other reports (9,13) that demonstrate a hierarchy of potency of peptides as tolerogens which reflects their immunogenicity *in vivo*. The finding that there is a loss of cytokine production by antigen-specific T cells following tolerance induction is consistent with a previous study on peptide-induced peripheral tolerance (28). However, there may be distinct mechanisms which operate in the induction of mucosal tolerance through the respiratory tract and this may be dependent upon the dose, the frequency and the nature of the antigen administered, as has been shown for oral tolerance (29).

Although the nasal administration of soluble peptide appears to induce a strong primary immune response (6), the functional outcome of this response is qualitatively different had the same peptide been administered in conjunction with adjuvant. We have previously suggested that mucosal tolerance to high-dose peptide induces a population of regulatory T cells which display an anergic phenotype *in vitro* (6). The cells remain in the peripheral circulation but can no longer act as classical T_h cells (15,16) but instead adopt an immunoregulatory role and function to modulate rather than promote antigen-specific immune responses. Furthermore, these regulatory T cells appear to be maintained in the peripheral circulation for long periods, as evidenced by their capacity to down-regulate T cell-dependent immune responses, such as cytokine secretion and DTH responses to Der p 1. Moreover, the regulatory T cells appear to be functional even 6 months after the original peptide treatment. This finding is in agreement with the stability of oral tolerance in mice where a single feed of protein to a naive animal can lead to life long antigen-specific T cell non-responsiveness (30).

Although antigen-specific T cell responses were modulated following tolerance induction, the levels of specific IgE antibody were not affected. The limitation to this study was that most immunizations were made in CFA which should bias for a T_h1 dominant immune response. Nevertheless, we have also observed similar findings when mice were also immunized under T_h2-type conditions. Therefore, the mucosally delivered peptide tolerogen appears to affect the function of $CD4^+$ T_h cells while B cell function in the short term does not appear to be modulated. These findings are consistent with studies which have examined immunological functions of patients who have undergone conventional allergen immunotherapy (31), where T cell functions were demonstrated to have been modulated without affecting the levels of specific serum IgE in the short term.

A feature of mucosal tolerance induced by nasally administered peptide is that a single peptide can inhibit responses to all epitopes on an antigen (13–17). In the model system studied here, the induction of tolerance to the immunodominant peptide of Der p 1 can down-regulate T cell responses to all four epitopes on the antigen when mice are immunized with the whole protein in adjuvant (15). However, it was possible to uncouple tolerance to the minor epitopes by immunizing tolerant mice with a peptide containing only the subdominant determinant, which is consistent with previous studies on peripheral T cell tolerance (8). However, it was possible to rescue a T cell response to a minor epitope of Der p 1 in mice that had been rendered tolerant to the dominant epitope and who had been previously immunized with the whole protein (Fig. 5). If such mice were immunized with the subdominant peptide in adjuvant, 3 weeks after the initial protein immunization, then the previously tolerant mice displayed equivalent responses to the subdominant epitope as controls.

Taken together, these observations suggest that the T cells specific for the minor epitopes are not deleted or anergized after nasal administration of the immunodominant peptide, but should be competent to respond to their epitopes following immunization with the protein. Yet this is not the case. Thus the intramolecular epitope suppression observed in our model is probably due to local suppression mediated by regulatory $CD4^+$ T cells through the recognition of linked epitopes on the same antigen. However, rather than killing the T cells specific for minor epitopes, we suggest that the regulatory T cells may merely block their growth. Once the immune response to the protein has waned, the regulatory T cells would return to a resting state since their epitopes would not be presented on APC. Thus when tolerant mice are

reimmunized with a peptide containing only the subdominant determinant, T cells specific for the epitope would be allowed to expand normally since they would no longer be under the control of the regulatory $CD4^+$ T cells. The phenomenon of linked suppression is not new and has been observed in a murine model of mAb-induced transplantation tolerance (18,19). Regulatory $CD4^+$ T cells induced by this tolerance protocol can mediate local suppression of allo-reactive T cells by linked recognition of antigen possibly on the same APC and can generate some bystander suppression to a third party antigen (18). Although we observed some inhibition of T cell responses to a bystander antigen in our model of peptide induced mucosal tolerance, the level of inhibition was not statistically significant.

We propose the following model to explain how intramolecular epitope suppression might be induced in mucosal tolerance. Following immunization of tolerant mice with the intact protein, APC in the draining LN will present peptides specific for the regulatory T cells and for those T cells recognizing the subdominant epitopes. The APC may therefore act as a bridge to bring the regulatory cells into close vicinity with the naive T cells of differing specificity. In such an environment the regulatory T cells may exert their inhibitory effects to other T cells either through the release of inhibitory cytokines or by negative signalling via direct membrane interactions. Alternatively, the regulatory T cells may compete for the local production of IL-2 by 'mopping up' excess IL-2 and thus prevent expansion of the naive T cells specific for the subdominant epitopes, as has been suggested by a human model of T cell anergy (32). The induction of bystander suppression in this model was not convincing, but this may be due to a problem in local antigen presentation, in that different APC may present the epitopes for the two antigens and these APC may be separated by a sufficient distance *in vivo* that enables the OVA-reactive T cells to escape the regulatory actions of the p111–139-specific T cells *in vivo*.

The observation reported here that T cell tolerance to i.n. peptide is long lasting may facilitate this route of antigen delivery in immunotherapy (33). Since a single immunogenic peptide alone can establish T cell tolerance by inducing a population of regulatory T cells, it may not be necessary to vaccinate with multiple T cell epitopes. In addition, further studies may help identify the molecular basis for tolerance induction and may also provide an insight into the functional properties of mucosal APC that make them effective in tolerance induction.

Acknowledgements

This work was supported by grants from the Wellcome Trust, UK, the Medical Research Council, UK and British Lung Foundation/Glaxo Wellcome (J. R. L.) and the National Health and Medical Research Council of Australia (W. R. T.).

Abbreviations

APC	antigen-presenting cell
CFA	complete Freund's adjuvant
Der p 1	*Dermatophagoides pteronyssinus* 1
DTH	delayed-type hypersensitivity
HDM	house dust mite
IFA	incomplete Freund's adjuvant
MBP	myelin basic protein
LN	lymph node
OVA	ovalbumin
PCA	passive cutaneous anaphylaxis
TGF	transforming growth factor

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Clin Exp Immunol 1997; 110:114–121

IL-5 production by allergen-stimulated T cells following grass pollen immunotherapy for seasonal allergic rhinitis

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(Accepted for publication 17 June 1997)

SUMMARY

Grass pollen immunotherapy for the treatment of seasonal allergic rhinitis ('summer hayfever') results in improvement in symptoms, a reduction in the early and late phase responses to allergen provocation and decreased tissue eosinophilia. Immunotherapy may act by altering the pattern of cytokine production by allergen-specific T cells from a 'Th2-type' (IL-4 and IL-5) profile to a 'Th1-type' (interferon-gamma (IFN-γ)) profile. We set out to determine whether clinical improvement following specific allergen immunotherapy is accompanied by reduced production of the pro-eosinophilic and archetypal 'Th2-type' cytokine, IL-5. Peripheral blood mononuclear cells (PBMC) were isolated from (i) 13 patients who had received 6 or 7 years' continuous conventional immunotherapy with timothy grass pollen (*Phleum pratense*); (ii) 14 patients who had received 3 or 4 years of conventional immunotherapy followed by 3 years of placebo treatment; (iii) 12 matched seasonal rhinitic patients who had never received immunotherapy; and (iv) 17 non-atopic normal controls. PBMC were stimulated with 20 μg/ml and 200 μg/ml *P. pratense* extract, or 10 μg/ml of *Mycobacterium tuberculosis* purified protein derivative (PPD), at 2×10^6 cells/ml and 5×10^6 cells/ml. IL-5 concentrations in culture supernatants collected after 6 days' culture were measured by ELISA. IL-5 production in response to stimulation with *P. pratense* extract was highly reproducible and was elevated in both of the immunotherapy treated groups and the untreated rhinitics relative to non-atopic controls ($P < 0{\cdot}005$ for each group relative to non-atopic controls, under each of the four conditions tested). However, no significant reduction was observed in IL-5 production when immunotherapy treated patients were compared with untreated rhinitic controls. Moreover, abrogation of the cutaneous late-phase responses to allergen following treatment was not associated with reduced IL-5 production by allergen-stimulated peripheral blood T cells. Reduced IL-5 production by peripheral blood T cells may not be necessary for immunotherapy to be effective. Local immunodulation of T cell responses may play a role in this form of treatment.

Keywords allergy immunotherapy T cell cytokines IL-5

INTRODUCTION

Although grass pollen immunotherapy is a highly effective treatment for seasonal allergic rhinitis [1–4], the exact nature of the mechanisms underlying its effects remains poorly defined. One possibility is that so-called 'blocking' IgG antibodies produced during immunotherapy may sequester allergen and thus prevent IgE-mediated activation of mast cells or basophils [5]. A reduction in the numbers of nasal metachromatic cells [6], presumed to be mast cells or basophils, has also been described following grass pollen immunotherapy. Allergen exposure in atopic subjects is characteristically associated with local expression of the Th2-type cytokines, IL-4 and IL-5 [7–9]. *In vitro* studies have demonstrated an association between allergic symptoms and production of IL-4 and IL-5 by allergen-specific T cells [10–12], and since allergen-specific T cells from non-atopic subjects are characterized by the expression of Th1-type cytokines such as interferon-gamma (IFN-γ) [10,11], it has been proposed that immunotherapy with aeroallergens may be associated with a shift in cytokine production

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by allergen-specific T cells from a Th2-type to a Th1-type profile. In support of this hypothesis, Secrist *et al.* [13] observed a reduction in IL-4 production by allergen-specific T cell lines derived from peripheral blood of immunotherapy treated patients relative to untreated controls, and we have previously reported increased expression of IFN-γ mRNA in skin and nasal biopsies of successfully treated patients after local allergen provocation [14,15]. A decrease in Th2-type and reciprocol increases in Th1-type cytokine production following high-dose antigen administration have also been described following insect venom desensitization [16–18]. A further feature of successful immunotherapy is a macroscopic inhibition of allergen-induced late-phase responses and an attendant reduction in the numbers of infiltrating eosinophils [15,19–21]. Since IL-5 acts specifically on eosinophils to promote their maturation, endothelial adhesion, activation and survival [22–24], and primes these cells for enhanced chemotactic response to C-C chemokines such as RANTES [25], we hypothesized that clinical improvement following grass pollen immunotherapy may be associated with a reduction in IL-5 production by grass pollen allergen-specific T cells in response to allergen exposure. We have previously shown that in short-term cultures of house dust mite-stimulated peripheral blood mononuclear cells (PBMC), IL-5 is secreted in a $CD4^+$-dependent manner and that the amounts produced are elevated in sensitized asthmatics and rhinitics relative to atopic and non-atopic controls without symptoms [12]. Thus, in order to test our hypothesis in the present study, we compared grass pollen allergen-induced IL-5 production by PBMC from immunotherapy treated patients, untreated rhinitics and non-atopic normal controls. To investigate further the specificity of any immunotherapy-induced changes in peripheral blood T cell function, we also compared responses to grass pollen and purified protein derivative (PPD; a ubiquitous antigen to which most subjects would be expected to be sensitized) in the same subjects.

SUBJECTS AND METHODS

Subject populations

Patients were selected for immunotherapy according to a history of severe summer hay fever that was poorly controlled by conventional medication. All patients had a >5 mm weal in response to skin testing with timothy grass pollen (*Phleum pratense*; Soluprick, ALK, Hørsholm, Denmark). Patients with other significant medical conditions (including a history of chronic asthma) were excluded. At the time of sample collection, 13 patients had received 6 or 7 years' continuous immunotherapy, and a further 14 patients had received 3 or 4 years' immunotherapy followed by 3 years' placebo treatment. The induction phase of immunotherapy was performed with twice weekly injections of Alutard SQ *P. pratense* over 6–8 weeks before progressing to monthly maintenance injections. Twelve matched atopic patients who had never received immunotherapy ('rhinitic controls') and 17 non-atopic subjects with a life-long absence of allergic symptoms were recruited as controls in this study. Both immunotherapy treated groups showed highly significant reductions in the sizes of cutaneous allergen-induced late-phase reactions and improvement in symptoms compared with untreated rhinitic controls (data not shown). Details of all subjects in the four clinical groups are shown in Table 1. Peripheral blood samples collected from these subjects were processed and cultured in the laboratory in a blinded fashion by a single investigator. This study was performed with the approval of the ethics committee of the Royal Brompton National Heart and Lung Hospital and the written consent of all participants.

Preparation of cells

PBMC were isolated from heparinized blood samples by density gradient centrifugation over Ficoll–Hypaque (Pharmacia, Uppsala, Sweden), washed twice in HEPES-buffered RPMI 1640 (Sigma, Poole, UK) and resuspended in RPMI 1640 (Sigma) supplemented with 5% human AB serum (Sigma), 100 U/ml penicillin/streptomycin (GIBCO, Paisley, UK) and 2 mM L-glutamine (GIBCO).

Cell cultures

For cytokine production, PBMC were resuspended at 2×10^6 and 5×10^6 cells/ml and incubated in 200 μl volumes (six replicates) in the presence of 20 and 200 μg/ml concentrations of *P. pratense* ('Aquagen' extract, kindly provided by ALK). Control cultures were incubated with medium or 10 μg/ml of *Mycobacterium tuberculosis* PPD (Evans Medical Limited, Leatherhead, UK) as a non-allergen control. All cultures were performed in 96-well flat-bottomed microtitre tissue culture plates (Nunc, Roskilde, Denmark). Supernatants were harvested from wells on day 6 and stored at −80°C pending measurement of cytokine concentrations. For proliferation, PBMC were resuspended at $0{\cdot}5 \times 10^6$ cells/ml in the presence of 20 and 200 μg/ml concentrations of *P. pratense*, and 10 μg/ml of PPD. Cellular proliferation was measured on day 7 by adding 0·5 μCi of tritiated methyl-thymidine (Amersham, Aylesbury, UK) per well for the last 16 h of culture, and assaying label incorporation by liquid scintillation spectroscopy.

Cytokine assays

IL-5 concentrations in culture supernatants were measured using a specific sandwich ELISA sensitive above 6·5 pg/ml, as previously described [26]. IFN-γ was measured using a commercially available ELISA (CLB, Amsterdam, The Netherlands) sensitive above 1 pg/ml.

Table 1. Clinical details of subjects

	IT 6–7 years	IT 3–4 years + placebo 3 years	Rhinitic controls	Non-atopic controls
Number	13	14	12	17
Age (years ± s.e.m.)	41·7 (± 3·8)	43·7 (± 2·5)	37·6 (± 3·7)	39·2 (± 3·9)
Gender (M:F)	8:5	7:7	8:4	11:6

IT, Immunotherapy.

Statistical analysis

The distribution of proliferative and cytokine responses was non-parametric, and between group comparisons were therefore performed using the Mann–Whitney *U*-test with the aid of a commercial software package (Minitab Inc., PA). The Bonferroni correction was applied for multiple comparisons. $P < 0{\cdot}05$ was considered significant.

RESULTS

PBMC proliferation in response to P. pratense *and PPD stimulation*

In order to determine the concentration or allergen extract required for optimal *in vitro* T cell activation, PBMC from 10 atopic donors were incubated with varying concentrations of *P. pratense*. Antigen-induced cellular proliferation increased in a concentration-dependent fashion (Fig. 1). The optimal dose of *P. pratense* required to illicit a proliferative response was between 20 and 200 μg/ml. *Phleum pratense*- and PPD-induced PBMC proliferation was statistically equivalent in both immunotherapy treated groups, the untreated rhinitic group and the non-atopic control group (Table 2).

Reproducibility of P. pratense*-induced IL-5 production*

To confirm that allergen-induced IL-5 production by peripheral blood T cells was reproducible, PBMC were purified from three *P. pratense*-sensitive atopic donors on three separate occasions (to ascertain interassay reproducibility). The first sample was also divided into two aliquots, each of which was then processed in parallel (to ascertain intra-assay reproducibility). IL-5 production under the conditions described was highly reproducible (Fig. 2), with all measurements in the order patient 3 >> patient 2 > patient 1. As can be seen from the individual data, there was good intra- and interassay reproducibility for measurements from each subject.

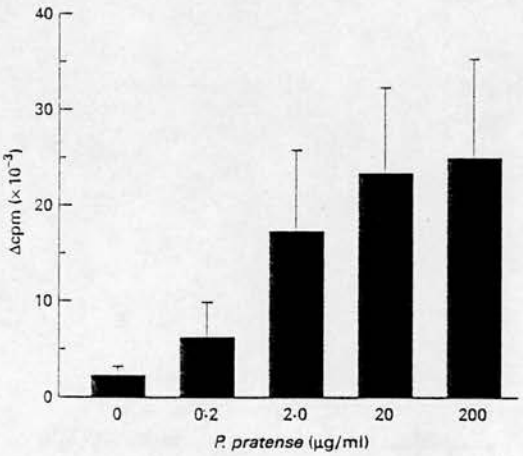

Fig. 1. Effect of allergen concentration *Phleum pratense*-induced peripheral blood mononuclear cell (PBMC) proliferation. Proliferation was assayed after 7 days. Data are from 10 atopic subjects and are represented for clarity as mean +s.d. Δct/min represents the counts per minute in antigen-stimulated cultures minus counts in unstimulated cultures (i.e. background).

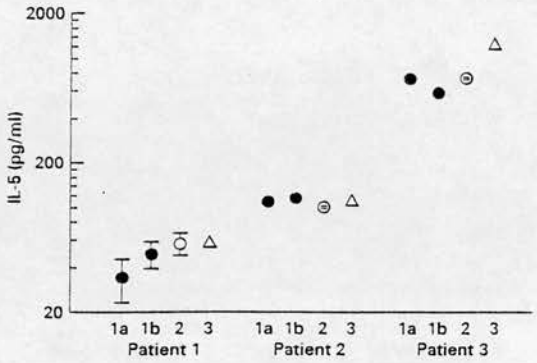

Fig. 2. Reproducibility of *Phleum pratense*-induced IL-5 production in three sensitized atopic subjects. Peripheral blood mononuclear cells (PBMC) were stimulated for 6 days at 5×10^6 cells/ml with 200 μg/ml *P. pratense*. IL-5 concentrations were measured in duplicate (shown as mean +s.d.): 1 (a and b), 2 and 3 represent data generated from blood samples collected on 3 different days. Data for 1a and 1b were generated from a single blood sample divided into two aliquots after collection, each of which was processed separately.

Effects of immunotherapy on P. pratense*-induced IL-5 production*

IL-5 production by PBMC stimulated with 20 μg/ml and 200 μg/ml *P. pratense* and 10 μg/ml PPD was determined in 13 immunotherapy treated rhinitics (6–7 years' active treatment), 14 rhinitics who previously received immunotherapy but were currently receiving placebo (3–4 years active, 3 years placebo), 12 rhinitics who had never received immunotherapy and 17 non-atopic normal controls. All measurements were performed on PBMC cultures performed at 5×10^6 cells/ml (Fig. 3) and 2×10^6 cells/ml (Fig. 4). Under all conditions tested, rhinitic groups (irrespective of whether they had previously received immunotherapy) were characterized by elevated IL-5 production compared with non-atopic normal controls. Nevertheless, no statistically significant differences were observed in allergen-induced IL-5 production between immunotherapy treated rhinitic groups and the untreated rhinitic group. This was also the case when the two separate groups of immunotherapy treated patients were combined and compared with untreated controls. Moreover, abrogation or inhibition of cutaneous late responses in immunotherapy treated patients was not associated with reduced allergen-induced IL-5 production by T cells within PBMC (Fig. 5). IL-5 production in PPD-stimulated cultures was lower than in the allergen-stimulated cultures. No significant differences were observed in IFN-γ production between any of the groups under any of the conditions tested. Comparison of ratios of IL-5 and IFN-γ produced in response to *P. pratense* or PPD (a prototypical Th1-type antigen) stimulation confirmed that IL-5 production by *P. pratense*-stimulated PBMC obtained from atopic subjects (immunotherapy treated and untreated), but not normal controls, occurred in the context of a Th2-like cytokine response (Table 2).

As further confirmation of the validity of our IL-5 measurements, significant positive correlations were observed between IL-5 production in response to 20 μg/ml *P. pratense* and 200 μg/ml *P. pratense*, when all atopic patients (immunotherapy treated and untreated) were analysed together ($r = 0{\cdot}88$, $P < 0{\cdot}0001$ at 5×10^6 cells/ml, and $r = 0{\cdot}92$, $P < 0{\cdot}0001$ at 2×10^6 cells/ml).

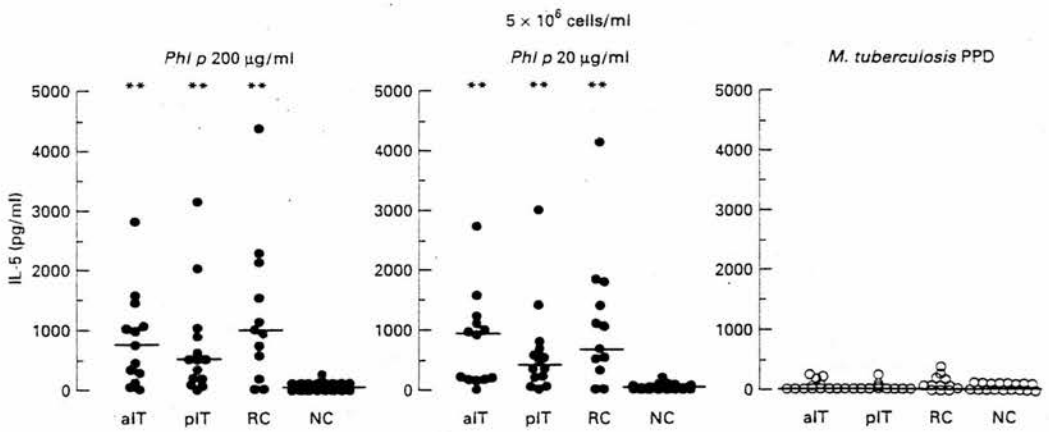

Fig. 3. Comparison of *Phleum pratense*- and purified protein derivative (PPD)-induced IL-5 production in immunotherapy treated rhinitic patients (aIT, pIT), untreated rhinitic controls (RC), and non-atopic normal controls (NC): aIT patients had received a total of 6–7 years' immunotherapy and were on active treatment at the time of the study. pIT patients had received a total of 3–4 years' immunotherapy but had received placebo treatment for the 3 years up to and including the time of the study. Peripheral blood mononuclear cells (PBMC) were cultured at 5×10^6 cells/ml. $^{**}P < 0{\cdot}005$ *versus* NC by Mann–Whitney *U*-test.

DISCUSSION

We have investigated the effects of specific allergen immunotherapy for the treatment of severe seasonal allergic rhinitis on the propensity of peripheral blood T cells to produce IL-5 in response to *ex vivo* stimulation with grass pollen allergen (*P. pratense*). We report that although *P. pratense*-induced IL-5 production by PBMC is elevated in appropriately sensitized rhinitic patients relative to non-atopic normal control subjects, treatment with a conventional allergen immunotherapy protocol, which resulted in a reduction in allergen-induced cutaneous late-phase responses and clinical improvement [3] in an overwhelming majority of patients, did not appear to decrease *P. pratense*-induced IL-5 production relative to untreated controls. Based on our findings, we speculate that a reduction in the ability of circulating peripheral blood T cells to secrete IL-5 on allergen stimulation is not a prerequisite for this form of immunotherapy to be effective.

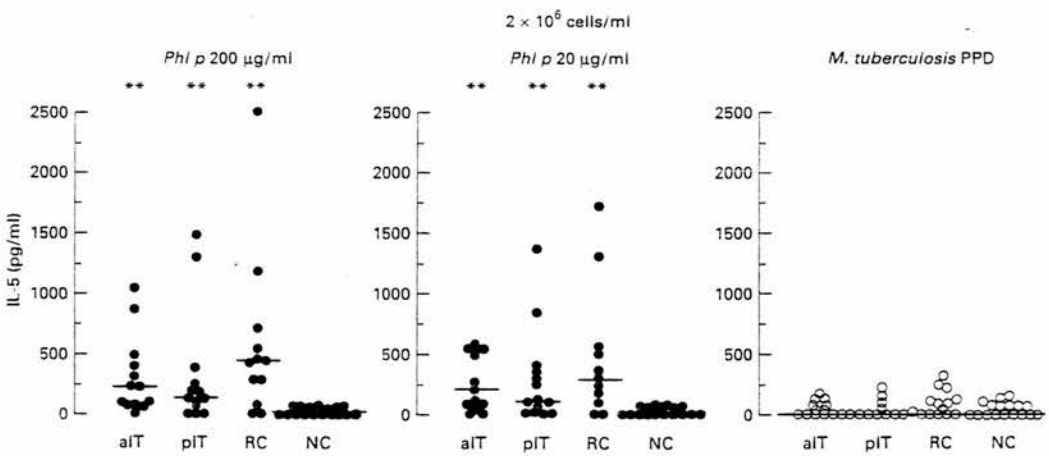

Fig. 4. Comparison of *Phleum pratense*- and purified protein derivative (PPD)-induced IL-5 production in immunotherapy treated rhinitic patients (aIT, pIT), untreated rhinitic controls (RC), and non-atopic normal controls (NC): aIT patients had received a total of 6–7 years' immunotherapy and were on active treatment at the time of the study. pIT patients had received a total of 3–4 years' immunotherapy but had received placebo treatment for the 3 years up to and including the time of the study. Peripheral blood mononuclear cells (PBMC) were cultured at 2×10^6 cells/ml. $^{**}P < 0{\cdot}005$ *versus* NC, by Mann–Whitney *U*-test.

Table 2 PBMC proliferation and cytokine production in response to grass pollen allergen and PPD stimulation. Cytokine data were generated from 6 day cultures, in which cells were cultured at 2×10^6 cells/mL. All data are shown as mean ± s.e.m.

	+ *P. pratense* (20 μg/ml)				+ *M. tuberculosis* PPD (10 μg/ml)			
	IT 7 years $n=13$	IT 4 years + placebo 3 years $n=14$	Rhinitic controls $n=12$	Non-atopic controls $n=17$	IT 7 years $n=13$	IT 4 years + placebo 3 years $n=14$	Rhinitic controls $n=12$	Non-atopic controls $n=17$
Proliferation (Δcpm)	48 608 (± 6995)	35 858 (± 5726)	41 300 (± 5603)	27 659 (± 4690)	94 584 (± 11 966)	114 416 (± 8530)	82 429 (± 8817)	92 804 (± 12 176)
IL-5 (pg/ml)	265·5 (±64)	279 (± 104)	460 (± 152)	15·2 (± 3·9)	57·1 (± 16)	46·1 (± 18·4)	108 (± 31·1)	29·8 (± 12·0)
IFN-γ (pg/ml)	411 (± 174)	544 (± 186)	747 (± 196)	1011 (± 174)	1502 (± 170)	1489 (± 107)	1380 (± 153)	1175 (± 159)
*IL-5/IFN-γ	2·21 (± 0·62)	1·44 (± 0·78)	1·062 (± 0·36)	0·05 (± 0·03)	0·08 (± 0·05)	0·04 (± 0·02)	0·12 (± 0·04)*	0·06 (± 0·03)

*Calculated as ratio of IL-5 to IFN-γ production within the same individual culture.

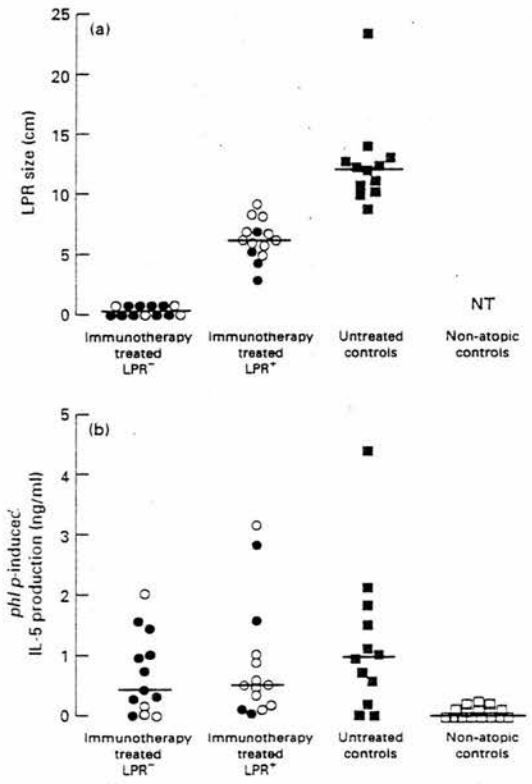

Fig. 5. Relationship between (a) size of cutaneous late-phase reactions (LPR) 6 h following intradermal injection with 30 BU of *Phleum pratense*, and (b) *P. pratense*-induced IL-5 production by peripheral blood mononuclear cells (PBMC) (200 μg/ml *P. pratense*, 5×10^6 cells/ml). ●, Patients who had received a total of 6–7 years' immunotherapy and were on active treatment at the time of the study; ○, patients who had received a total of 3–4 years' immunotherapy but had received placebo treatment for the 3 years up to and including the time of the study. NT, Not tested.

The association between the production of Th2-type cytokines such as IL-4 and IL-5 by allergen-specific T cells and the presence of atopic diseases, such as asthma or allergic rhinitis, is well established. Although many of these studies have been based on analyses of allergen-specific T cell lines or clones [10,27], a number of more recent studies have also demonstrated elevated IL-4 [11] or IL-5 [12] production by peripheral blood T cells from atopic subjects stimulated by allergen in short-term primary cultures of PBMC. Data generated in our group [12] have shown that IL-5 production by allergen-stimulated PBMC is elevated in symptomatic atopic patients (asthma or rhinitis) relative to atopic and non-atopic subjects without any symptoms, and that IL-5 synthesis in this system is dependent on the presence of $CD4^+$ but not $CD8^+$ T cells. We chose to use this primary culture system to investigate the effects of immunotherapy on IL-5 production by allergen-specific peripheral blood T cells, because it is highly reproducible and generates data with clear cut differences between rhinitic patients and normal controls. Moreover, the sole stimulus in these cultures is allergen extract: cells are not subject to expansion in IL-2 or stimulation with non-specific mitogens, which may distort the allergen-specific T cell repertoire or unphysiologically augment cytokine synthesis.

In the present study, we also observed T cell proliferative responses and IFN-γ (but not IL-5) production in normal controls. These findings are consistent with those of Imada *et al.* [11], who recently demonstrated that non-atopic individuals mounted *in vitro* T cell responses to grass pollen, although in contrast to atopics, these subjects' T cells favoured IFN-γ, but not IL-4 production under these conditions. While it may have been preferable to perform the analyses described before and throughout the immunotherapy course, this was impracticable in the present study. Furthermore, we took a number of precautions to standardize measurements in different subjects. First, in patients currently receiving immunotherapy (or placebo treatment), all blood samples were collected 3–4 weeks after the last injection of allergen. Second, all blood samples were processed 1–2 h after collection, and cultured with identical batches of allergen extract and culture medium supplements (AB+ serum, L-glutamine and antibiotics). The reproducibility of these assays was examined in initial experiments performed on three subjects on three separate occasions (Fig. 1) and subsequently confirmed in study subjects by virtue of the strong linear correlation observed between IL-5 measurements made using two different concentrations of allergen extract.

Although studies performed on peripheral blood cells have yielded invaluable information, the relationship between cytokine secretion by allergen-specific T cells within peripheral blood and the target organ remains poorly defined. Previous work from our group has shown that exposure of the nasal mucosa to grass pollen is associated with recruitment and activation of T cells and eosinophils [28] and preferential expression of mRNA for the Th2-type cytokines, particularly IL-4 and IL-5 [15]. The principle cell source of IL-5 was the T cell (83·2%), with minor contributions from mast cells (11·3%) and eosinophils (5·4%) [8]. We also examined the influence of immunotherapy on these changes in the target organ. Immunotherapy was not associated with statistically significant reductions in the numbers of IL-4-expressing cells, although there was a trend towards a decrease in IL-5 expression [15], and increased numbers of IFN-γ and IL-12 mRNA-expressing cells were observed [14,15,29]. Moreover, IFN-γ expression within the nasal mucosa was found to correlate with clinical improvement following immunotherapy [15]. More recent functional studies suggest that the ability of allergen-specific T cells to secrete IL-5 is markedly up-regulated at sites of allergen exposure, since purified bronchoalveolar lavage T cells (collected 24 h after segmental allergen challenge) stimulated with allergen and antigen-presenting cells secreted significantly higher amounts of IL-5 than equivalent numbers of peripheral blood T cells from the same subjects (unpublished observations). These observations suggest that allergen-specific T cells may undergo differentiation into a cytokine-producing 'effector' phenotype following *in vivo* allergen exposure and recruitment to tissues.

Although peripheral blood T cells from atopic subjects are predisposed to secrete IL-4 and IL-5 in the context of a Th2-type cytokine profile on stimulation with allergen (but not with bacterial antigens such as *M. tuberculosis* PPD, which favour IFN-γ production [30,31], see Table 2), the cytokine-producing phenotype of these cells retains some flexibility, since production of IL-4 and IL-5 *in vitro* in response to activation by allergen can be

inhibited by IL-12 and IFN-γ [32–34]. We hypothesize that expression of cytokines such as IL-12 and IFN-γ at the sites of allergen exposure plays a key role in regulation of T cell cytokine responses following immunotherapy, by interceding in up-regulation of local IL-5 (and possibly IL-4) production. This hypothesis is consistent with our findings of IL-12 and IFN-γ mRNA expression in biopsies of allergen-induced late-phase reactions in patients who have received immunotherapy [14,15,29]. Although the numbers of IL-5 mRNA-transcribing cells in these biopsies did not change with immunotherapy, *in situ* hybridization does not quantify the amount of mRNA produced by a particular cell, and it is quite possible that in T cells in which IL-5 production has been inhibited by IL-12/IFN-γ, complete extinction of IL-5 transcription may not occur.

There have been relatively few previous studies of the properties of allergen-specific T cells following conventional immunotherapy with aeroallergen extracts. Secrist *et al.* [13] showed that allergen-specific T cell lines propagated from the peripheral blood of immunotherapy treated atopic patients and stimulated with mitogen secreted markedly less IL-4 compared with a group of untreated atopics. However, this study included patients that were sensitive to both house dust mite and grass pollen, and who had received immunotherapy for differing lengths of time. Moreover, this study employed culture techniques that necessitated expansion of cells in IL-2 and stimulation with non-specific mitogens. Similar findings have also been reported following rush desensitization for wasp and bee venom anaphylaxis [16–18]. In contrast, we did not identify either a reduction in IL-5 or an increase in IFN-γ production in PBMC cultures following immunotherapy, despite use of validated methods in a blinded controlled clinical study. However, our previous findings of 'immune deviation' within the target organ (i.e. the nose), with a non-significant trend for a reduction in IL-5 expression and significant increases in IFN-γ and IL-12 mRNA expression, support the concept that local alterations in T lymphocyte responses upon subsequent allergen exposure may underlie the clinical benefits following allergen immunotherapy.

In conclusion, we have investigated the effects of immunotherapy on the capacity of peripheral blood allergen-specific T cells (within PBMC) to produce IL-5 on *ex vivo* stimulation with allergen extract. Our observations suggest that a profound alteration of allergen-specific T cell function in the peripheral circulation may not be a requirement for the clinical efficacy of this form of treatment, and support the concept that local immunomodulation may be at least partly responsible for the effects of immunotherapy.

ACKNOWLEDGMENTS

This study was supported by a grant from the National Asthma Campaign. We would like to thank ALK for providing allergen extracts for immunotherapy and cell culture.

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2.3 ANTIGEN MEDIATED MODULATION OF T CELL FUNCTION

2.3.4 INDUCTION OF REGULATORY (INHIBITORY) CELLS

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Reprinted from Nature, Vol. 300, No. 5891, pp. 456–458, 2 December 1982

A human suppressor T cell clone which recognizes an autologous helper T cell clone

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In contrast to the many reports of IL-2 dependent proliferating, helper or killer cell clones[1], there is only a single report of an IL-2 dependent suppressor cell clone[2], in the mouse. However by the same cloning procedures used to generate human helper cells[3], suppressor cell clones to influenza virus could not be generated, and so another strategy was used. Jerne's network hypothesis[4] proposes that immune regulation results from lymphoid cell receptors recognizing determinants on other lymphoid cell receptors. If this is the case it should be possible to generate regulatory T cell clones against other T cells and we report here the generation of an autologous suppressor cell clone which recognizes and inhibits the function of a human helper T cell clone. Such an autologous suppressor cell clone provides a new approach to understanding the pathways and molecular events involved in immune regulation. Mutual stimulation of the suppressor cell clone and the target helper cell clone in the absence of back stimulation, provides direct experimental evidence for the existence of interactions between T cell receptors, and thus suggests that the specificity of the suppressor cell clone is for the antigen receptor of the helper cell.

Human immunology, unlike mouse immunology, is limited by restricted access to genetically uniform cells. In order to circumvent this problem, all the experiments reported here were performed with cryopreserved cells from a single individual. T lymphocyte clone 6 (TLC 6) is a helper cell which recognized influenza A matrix protein in conjunction with HLA-DR1[3–6]. It was used to generate anti-idiotype suppressor cells because it grows well in culture, and its helper activity is easy to measure *in vitro* using the influenza virus specific antibody response and assay system developed by our colleagues, Callard, Beverley and Zanders[7–9], using either the cells themselves, or its antigen specific helper factor.

To stimulate autologous peripheral blood cells (PBL) with irradiated TLC6, a 10-day mixed lymphocyte culture was performed. The resulting blasts were purified on Ficoll–Hypaque and restimulated with irradiated TLC6. The ensuing lymphoblasts purified on a Percoll gradient three days later, and cloned as described in Table 1. Approximately 60 clones were grown up, and tested for their specificity. For simplicity, proliferation was used as the criterion of responsiveness, and cells from individual clones were cultured with irradiated autologous PBL and either the immunogen TLC6, another helper clone from the same donor HA1.9, influenza A virus, or just PBL alone. After 72 hours, cultures were pulsed with tritiated thymidine and cells were collected 16 hours later. Several patterns of reactivity were seen. Clones from these experiments were termed AC1 to 60. The most common response was to influenza virus (for example clone AC10), which could have been still present on the surface of washed TLC6 even after one week's culture. Other clones showed no detectable response to any of the stimuli used (for example AC57). This is paradoxical, as only activated cells respond to TCGF and can be cloned[1]. It seems likely that these clones were responding to TLC6, but the response was not detectable by the assay used. Clone AC34 is presumably recognizing an antigen on PBL (or serum), whereas clone AC5 is recognizing an activated cell antigen. The specificity of clones AC19 and AC32 is not known.

The most relevant clone, chosen for further analysis, was clone AC50. This proliferated in response to TLC6, but not other clones, PBL or influenza virus. Thus it seemed to be specific for TLC6, and was a candidate for an anti TLC6 clone. In the absence of any antibodies which unambiguously define human T-cell receptors, we used a functional assay to analyse the specificity of clone AC50 further.

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Table 1 Induction of anti-TLC 6 clones

Clone no.	Antigen					
	6 + PBL	HA1.9 + PBL	PBL	Flu + PBL	Medium	TCGF
AC5	1,249 ± 94	5,469 ± 1,595	44 ± 10	75 ± 21	39 ± 16	11,450 ± 750
AC10	43 ± 20	57 ± 16	54 ± 16	4,676 ± 404	31 ± 11	5,063 ± 162
AC19	2,534 ± 367	2,794 ± 366	2,457 ± 326	31 ± 8	15 ± 4	3,164 ± 243
AC22	1,518 ± 247	24 ± 2	46 ± 11	2,478 ± 35	34 ± 4	8,695 ± 622
AC34	1,801 ± 360	2,255 ± 304	1,655 ± 68	3,005 ± 912	29 ± 7	6,243 ± 221
AC50	2,928 ± 94	32 ± 13	36 ± 10	54 ± 8	21 ± 3	9,949 ± 216
AC57	23 ± 4	22 ± 5	24 ± 4	30 ± 5	21 ± 2	9,720 ± 506
AC58	4,550 ± 570	3,711 ± 446	7,508 ± 1,620	5,801 ± 1,494	26 ± 3	9,097 ± 208

Peripheral blood lymphocytes (PBL, 5×10^5 per ml) were cultured with irradiated (2,500 rad; ^{137}Cs) TLC6 (5×10^5 per ml) in RPMI 1640 supplemented with 10% pooled A$^-$ serum in a 24 well plate (Costar). Both the responder (PBL) and stimulator (TLC6) cells were derived from the same donor. After 10 days of primary culture the viable cells were isolated on Ficoll–Hypaque and blast cells (10^5 per ml) were restimulated with irradiated TLC6 (10^5 per ml) in the presence of autologous irradiated PBL (5×10^5 per ml). Three days after restimulation the lymphoblasts were enriched on a discontinuous Percoll (Pharmacia) density gradient (35–40%) and plated at 0.3 cells per well in sterile 60 well Microtest II trays (Falcon) in the presence of T cells growth factor (TCGF or Interleukin 2) irradiated stimulator cells (TLC6; 5×10^3 per well) and autologous irradiated PBLs (5×10^3 per cell) as a source of antigen presenting cells (APCs). TCGF was prepared from PBL (1×10^6 per ml cultured with PHA (0.1%) in the presence of 1% autologous serum for 48 hours as described elsewhere[3,11]. The wells that showed positive growth (3.4% of the total number of blast cell seeded) at 7 days were transferred to 96 well flat bottom microtire trays (Falcon) and cultured with irradiated TLC6 (5×10^4 per well) and autologous irradiated PBL (5×10^4 per well) in the presence of TCGF. Following a further 7 days in culture, the clones were transferred to 24 well trays containing TCGF, irradiated TLC6 (5×10^4 per well) and autologous irradiated PBL (1×10^6 per well). In the preliminary screen clones were examined for antigen specificity in a 72 hour array by using titrated methyl thymidine (^{3}H-TdR; NEN). A 1:200 dilution of individual clones from 24 well trays were cultured with autologous irradiated PBL (25×10^3 per well) and irradiated TLC6 (5×10^3 per well) in a total volume of 200 µl. Clones T cells from the same donor but with a different antigen specificity (HA1.9) together with autologous PBL, PBL alone or influenza A virus (A Texas/1/77; 5 haemagglutinating units (HAU) per ml) in the presence of PBL were added as controls. After 72 hours, cultures were pulsed with 1.0 µCi of ^{3}H-TdR and cells were collected 16 hours later. Incorporation of ^{3}H-TdR was measured by liquid scintillation spectroscopy. Results are expressed as mean c.p.m. ± s.e.m. We have checked AC50 for its reactivity with other helper clones from the same individual but with different fine specificities. It did not react with 2 other helper clones (HA1.7, HA1.9) as well as other autologous clones (37, 53, 72).

Table 2 Anti-idiotypic activity of clone AC50

Clone no.	Antigen: Clone AC50* (5×10^2)	Clone AC50* (1×10^3)	Clone AC50* (5×10^3)	Medium	TCGF
6	820±124	1,160±169	2,274±322	17±4	3,472±313
6+E⁻*	3,413±369	4,074±254	5,328±590	31±16	
6+E⁻+Flu	10,311±2,404	8,902±866	7,386±1,063	9,360±495	
37	43±9	59±8	65±16	35±3	8,551±1137
37+E⁻	38±8	71±18	32±4	53±13	
37+E⁻+Flu	13,569±1,861	9,978±1,348	11,703±1,018	14,325±2,543	
53	27±4	35±12	17±6	25±6	6,217±491
53+E⁻	33±5	18±2	24±2	12±1	
53+E⁻+Flu	4,261±434	4,954±129	6,203±631	4,829±686	
72	23±1	8±2	19±5	20±5	4,142±474
72+E⁻	41±14	26±3	13±2	29±3	
72+E⁻+Flu	7,443±1035	7,248±131	8,130±1076	7,055±360	
HA1.7	17±2	15±2	10±3	31±6	3,447±122
HA1.7+E⁻	33±1	43±8	18±2	12±1	
HA1.7+E⁻+Flu	3,625±329	4,288±372	4,100±105	3,814±261	
CTLL	58	49±11	115±17	73±14	10,361±1,895
CTLL+E⁻	101	146±23	742±109	60±8	
CTLL+E⁻+Flu	20,183	17,081±3,036	15,622±1,388	18,762±2,573	

Various numbers of irradiated AC50 cells (5×10^2, 1×10^3 and 5×10^3 per well) were cultured with a panel of influenza A virus-specific TLCs (5×10^3 per well) in the presence or absence of autologous irradiated sheep erythrocyte (SRBC) rosette negative (E^-) cells (5×10^3 per well) and Influenza A virus. E^- cells were separated from those cells forming rosettes with AET (aminoethyliisoethiouronium bromide hydrobromide)-treated SRBC by centrifugation over Percoll (1.080 g ml^{-1}). Proliferative responses were determined by the incorporation of ^{3}HTdR as described in the legend to Table 1. Results are expressed as mean c.p.m. (±s.e.m.). CTLL, influenza specific T lymphocyte line.

* E^- and AC50 irradiated 2,500 rad.

We argued that if receptors on AC50 recognized anti-TLC6 receptors, then TLC6 might be stimulated to respond to irradiated AC50 *in vitro*, as its receptors would be bound. Thus a variety of influenza specific clones (all from the same donor) were stimulated with irradiated clone AC50, or AC50 plus irradiated E^- cells (T depleted cells) as a source of antigen presenting cells. As a control the response to AC50, PBL and influenza virus was assayed. As shown in Table 2, all the clones responded to influenza virus and TCGF, but only TLC6 responded to AC50, with or without the addition of irradiated E^- cells. This result is compatible with the notion that AC50 is an auto anti-idiotype clone, that is, recognizes antigen-specific receptors on TLC6. The response in the absence of additional antigen presenting cells (APC) is of interest, implying that the reaction between TLC6 and AC50 may not require antigen presenting cells. However, a few APC, which had not adhered to the plastic in the week of culture since irradiated PBL were added could still have been present, and this question needs to be analysed in detail. It is of interest that Infante *et al.*[10] recently found that anti-idiotype antisera stimulate mouse T cell clones to proliferate even in the absence of added antigen presenting cells, in analogy to the results shown here with anti-idiotype T cells.

Table 3 Lack of back stimulation in mixed cultures of AC50 and TLC6

Responder cells	Stimulation (c.p.m.±s.e.m.): AC50	6	AC50+6	FluA	Medium	TCGF
CTLL	43	17	31	15	23	6,503
	±7	±4	±9	±2	±6	±294
CTLL+E⁻	12	21	57	14,689	27	—
	±5	±5	±8	±2,229	±4	

Irradiated AC50 (5×10^3 cells per well) and TLC6 (5×10^3 cells per well) were cultured alone or together with an influenza specific cultured T lymphocyte line (not the same as in Table 2) CTLL (5×10^3 cells per well) in the presence or absence of autologous irradiated E^- cells. As controls CTLL cells were cultured with and without irradiated E^- cells and influenza A virus. Proliferative responses were measured and results expressed as described in Table 1.

The results in Table 2 indicate that AC50 and TLC6 interact in a specific manner. But they do not conclusively indicate that the reaction is anti-receptor. An alternative interpretation is that the proliferation of TLC6 is due to back stimulation[3,11] with irradiated AC50 recognizing TLC6, and it is the release

Table 4 Cloned auto (anti-idiotypic) suppressor T lymphocytes

Helper T cells	Stimulus: Autol. E^- (5×10^5 per ml)	A/Texas/1/77 (0.5 HAU per ml)	Suppression (AC50 per ml)	Response: Anti-influenza A virus antibody (ng ml^{-1})
E⁻*	−	−	−	0
E⁻	−	+	−	0
−	+	−	−	0
−	+	+	−	4±1
E⁻	+	+	−	145±26
TLC6†	−	−	−	0
+	+	−	−	0
+	+	+	−	208±22
+	+	+	50	10±2
+	+	+	250	3±1
+	+	+	500	0

Cloned helper T cells (TLC6; 2.5×10^3 per ml) or E^- cells (5×10^5 per ml) were cultured with autologous E^- cells in the presence of influenza A virus (A/Texas/1/77; 0.5 HAU ml^{-1}) in RPMI 1640 supplemented with 10% horse serum in 96 well round bottom microtitre trays. TLC AC50 cells were added at 50, 250 and 500 cells per ml at the initiation of the cocultures each of 200 µl. After 6 days incubation at 37 °C, triplicate cultures were washed and recultured in 150 µl of RPMI 1640 supplemented with 5% fetal calf serum (FCS). Supernatants were collected after 24 h and assayed for anti-A/Texas/1/77 antibody using a solid phase enzyme immunoassay. Antibody production was determined by reading absorbance at 405 nm of the transformed substrate of alkaline phosphatase[9]. The actual amount of antibody (ng ml^{-1}) ± standard error of triplicate cultures. Results are expressed as the mean (ng ml^{-1}) and for each triplicate the s.e. was <20%. Background responses of TLC6, E^- and E^- cultured alone or with influenza A virus were measured. * 5×10^5 ml^{-1}; † 2.5×10^3 ml^{-1}.

of lymphokines from irradiated AC50 which causes TLC6 to incorporate thymidine. This possibility was investigated, using a cultured (not cloned) T lymphocyte line (CTLL) which responds well to TCGF (Table 3). A mixture of irradiated AC50 and TLC6 could not stimulate CTLL to proliferate (31 c.p.m.) in contrast to TCGF (6,503 c.p.m.), as shown in Table 3. There was no effect of AC50 and TLC6 even in the presence of E- cells.

Thus the induction of thymidine incorporation in TLC6 by AC50 was not due to back stimulation, and thus the bidirectional nature of the TLC6–AC50 interaction and its specificity for TLC6, but no other autologous clones would appear to indicate that AC50 recognizes antigen-specific receptors on TLC6. Because the reaction is autologous, and specific for TLC6 it cannot be directed against MHC or differentiation antigens. The criteria we have used to define the anti-idiotype nature of clone AC50 (that is, specificity, stimulatory effects) are the same as used for anti-idiotypic antisera (discussed in refs 10, 12).

The effects of clone AC50 on the helper response of TLC6 were assessed *in vitro* (Table 4). Very small numbers of AC50 diminished the response. Only 10 clone AC50 cells inhibited the helper response of 500 TLC 6 (to 100,000 E^- cells in a 200 μl culture volume) by 95%. Fifty cells of AC50 per culture abolished the response altogether. These results indicate that AC50 is a potent suppressor cell. The mechanism of suppression is unknown, but it is unlikely, due to the ratio of cells used (1 suppressor to 50 helpers) and the capacity of irradiated AC50 to induce TLC6 to incorporate thymidine (Table 2), that AC50 is cytotoxic for TLC6. The mechanism of suppression remains to be elucidated, but because it occurs at the clonal level should be amenable to precise analysis.

These results indicate that as in the mouse, it is possible to generate a human specific suppressor T cell clone dependent on IL-2. In contrast to the mouse suppressor cell clone, specific for antigen, the specificity of the human suppressor clone was for the immunizing cell (and not other autologous helpers). The reciprocal stimulation, in the absence of back stimulation, suggests that the target of AC50 is the receptor for antigen of TLC6. This is a direct experimental test of the 'idiotype network' at the T cell level. The potency of the suppressor effect suggests that this type of suppressor cell may be of major importance in immune regulation.

The generation of suppressor T cells recognizing helper cell receptors resembles work reported by Binz and Wigzell, who injected antigen-specific lymphoblasts into autologous mice[13]. Specific unresponsiveness resulted, which was mediated by either T killer, suppressor or helper cells, or B cells[13]. Conceptually the results are analogous, but the experiments reported here were performed entirely *in vitro*, and at the clonal rather than the population level. There are reports of suppressor cell pathways to the haptens nitrophenyl (NP) or azobenzene arsonate (ABA) for delayed hypersensitivity which involve second order suppressor cells, which recognize idiotype bearing cells[14]. However, the final suppression was not idiotype specific[15]. These experiments were also performed at the population level.

By possessing a clone of suppressor cells (AC50), specific for a clone of helper cells (TLC6), we have a powerful system for analysing the mechanism of T cell suppression. There is no reason to believe that the principle demonstrated here will not apply with other clones. Thus, situations with undesirable clones of helper (or killer) cells such as autoimmunity or perhaps even leukaemia may be controllable by suppressor cells of this type, generated *in vitro*.

This work was supported by US Office Naval Research Contract N000-14-77-C-0748 USPHS, and the Imperial Cancer Research Fund. We thank Drs Woody, Eckels and Lake for their helpful assistance in this work, Philippa Wells for typing the manuscript and Dr M. C. Raff for his helpful comments on the manuscript.

Received 30 July; accepted 28 September 1982.

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Inhibition of human T-cell responses to house dust mite allergens by a T-cell receptor peptide

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Recent analysis of the usage of T-cell receptor (TcR) β chain variable region (Vβ) gene elements by house dust mite (HDM)–reactive T cells from an atopic donor suggested that TcR-Vβ3 gene products may form a major component of the human T-cell repertoire reactive to this common allergen. In this study a peptide analog of the TcR-Vβ3 complementarity determining region 2 (CDR2) is shown to inhibit the polyclonal human T-cell response to HDM; this effect is specific because inhibition is dependent on the presence of Vβ3+ T cells. This experimental approach has been used to determine whether the pattern seen in T-cell clones derived from one atopic donor reflects TcR-Vβ usage in the polyclonal response to allergen in the general population. Inhibition of more than 50% of the polyclonal response to allergen by Vβ3-CDR2 peptide was observed in 16 of 21 donors tested, suggesting that TcR-Vβ3 gene usage may form a major component of the human HDM repertoire and as such offer a suitable target for T cell–directed specific immunotherapy in HDM-allergic individuals. Depletion of CD8+ T cells abolishes peptide-mediated inhibition of CD4+ T-cell proliferation to HDM, suggesting that induction of a CD8+ regulatory T-cell subset by the CDR2 peptide may modulate HDM-specific allergic T-cell responses. (J ALLERGY CLIN IMMUNOL 1994;94:844-52.)

Key words: House dust mite, T cells, TcR-CDR2 peptide

CD4+ T lymphocytes from atopic individuals recognize processed fragments of allergens from house dust mite (HDM) bound to major histocompatibility complex (MHC) class II molecules. These T cells become activated to induce and regulate the effector mechanisms, including the production of IgE and the activation of inflammatory cells, of the allergic immune response.[1, 2] The capacity to specifically downregulate antigen-mediated stimulation of allergen-reactive CD4+ T cells from atopic individuals may provide a means to alleviate the symptoms of allergic disease without the undesirable and long-term side effects associated with the nonspecific immunosuppressive therapies currently in use. Evidence for the

Abbreviations used

CDR2:	Complementarity determining region 2
EAE:	Experimental allergic encephalomyelitis
HDM:	House dust mite
IL-2:	Interleukin-2
MHC:	Major histocompatibility complex
MTSE:	*Mycobacterium tuberculosis* soluble extract
PBMCs:	Peripheral blood mononuclear cells
TcR:	T cell–specific antigen receptor
Vβ:	Variable region of the β chain of the T-cell receptor

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Dr. Jarman and Dr. O'Hehir are recipients of a Rodney Porter Studentship and Wellcome Senior Fellowship in Clinical Science, respectively. This research was funded by grants from the Wellcome Trust.

Received for publication Sept. 29, 1993; revised Apr. 1, 1994; accepted for publication Apr. 26, 1994.

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0091-6749/94 $3.00 + 0 1/1/57587

recognition of a limited array of HDM epitopes or the recruitment to the disease site of T cells expressing T cell–specific antigen receptor (TcR) with shared characteristics would allow these cells to be specifically targeted and facilitate the development of TcR-based immunotherapy.

Recent experiments in our laboratory have demonstrated that T lymphocytes from an atopic individual with allergy to HDM show a bias in usage of TcR-Vβ gene elements. This was demonstrated with anchor polymerase chain reaction analysis of T-cell clones derived from peripheral blood that were reactive with HDM. Of 10 clones analyzed, six different TcRαβ chain combinations were identified of which three, or 50%, used Vβ3 and the others used Vβ6.7a, Vβ9, and Vβ21. This TcR-Vβ gene usage appears to persist with time because when the same individual was tested over a period of several years, the HDM-reactive TcR repertoire appeared unchanged,[3] and dominant long-lived clones could still be detected in the periphery 6 years later. If the same pattern of restricted Vβ gene usage observed in T-cell clones derived from a single atopic individual is representative of the polyclonal response observed in the majority of HDM-allergic individuals, it may be possible to design specific immunotherapy for HDM-allergic individuals by targeting allergen-reactive T cells with restricted Vβ usage. In an earlier study analysis of TcR-Vβ usage by HDM-reactive T cells at the population level was limited by the requirement to clone, confirm antigen specificity, and perform anchor polymerase chain reaction and sequence analysis of the TcR repertoire of multiple T-cell clones derived from a large number of individuals. We have circumvented this approach by using an experimental design that permits analysis of Vβ3 usage in the polyclonal response to HDM.

The T cell–specific antigen receptor is a member of the immunoglobulin superfamily of molecules, and the variable regions of both the α (Vα) and β (Vβ) chains of the TcR have four hypervariable loops, also known as complementarity determining regions (CDR1, 2, 3, and 4), which form the antigen-binding regions.[4] CDR3 appears to be the most diverse of these regions and is believed to be involved in antigen contact. The CDR1 and CDR2 regions are implicated in MHC binding.[5] The fourth hypervariable region, CDR4, appears to participate in superantigen binding.[6] Recent experiments in rodent models suggest that these regions of the TcR not only serve to promote antigen and MHC binding but may also (in particular the Vβ-CDR2 region) serve as an antigenic determinant or recognition site for regulatory T cells.[7-9]

"Disease-associated" T cells that preferentially use selected TcR gene products have been identified in animal models of experimental allergic encephalomyelitis (EAE). Encephalitogenic T cells in both rat and mouse EAE express predominantly TcR-Vβ8.2 genes, and immunization with a synthetic peptide corresponding to the CDR2 of TcR-Vβ8.2 prevents clinical signs of disease.[7-12] We have tested the capacity of a CDR2 peptide derived from the human TcR-Vβ3 to modulate the polyclonal human T-cell response to HDM allergen. We demonstrate that this peptide specifically inhibits the antigen responsiveness of Vβ3-expressing T cells, and we have used this observation to study the contribution of Vβ3 T cells to the HDM response in the population as a whole. A panel of HDM-primed, genetically unrelated individuals was chosen for this study. Inhibition of more than 50% of the polyclonal response to HDM by CDR2 peptide was observed in 75% of all individuals tested; less than 10% of donors showed no inhibition of CDR2. These data suggest that TcR-Vβ3 usage may form a major component within the HDM-reactive T-cell population and may thus provide a target for immunotherapy. Studies into the mechanism of CDR2 inhibition are also presented.

METHODS

Patients

The atopic status of patients with HDM allergy was defined by the presence of a positive skin prick test response[13, 14] (wheal diameter ≥3 mm) to *Dermatophagoides pteronyssinus* and *D. farinae* extracts in the presence of a negative saline control. Sixteen HDM-allergic atopic subjects, aged 15 to 60 years, were included in the study. All of these individuals had clinical symptoms of HDM allergy (perennial rhinitis and/or asthma) and had grade III or IV serum-specific IgE for HDM as measured by RAST.

Nonatopic status was defined in a subject who was free of symptoms by the absence of positive skin prick test responses to a panel of common environmental allergens (*D. pteronyssinus, D. farinae*, mixed grass pollen, birch pollen, plane tree, nettle, *Aspergillus fumigatus, Cladosporium herbarium*) in the presence of a positive wheal response to histamine hydrochloride (1 mg/ml) of 3 mm or greater and a negative response to a saline control. Five normal healthy nonatopic individuals were used for this study. All had negative skin prick test results and normal total IgE levels.

Antigens

Lyophilized, unfractionated extracts of *D. pteronyssinus* and *D. farinae* were generously provided by Dr. A. Wheeler (SmithKline Beecham PLC, Brentford, Middlesex, U.K.) and Dr. H. Lowenstein (ALK Laboratories, Horsholm, Denmark), respectively. *Mycobacterium tuberculosis* soluble extract (MTSE) was a kind gift from the TB and Related Infections Unit at Hammersmith Hospital, London.

Peptides

The CDR2 40-61 (GLGLRLIYFSYDVKMKEKGDI) peptide used overlaps the predicted region for the second hypervariable region of the TcR-Vβ3 expressed by the CD4+ T-cell clone HA1.7. Influenza virus hemagglutinin peptide HA 307-319 (PKYVKQNTLKLAT), keratin peptide K1-9 (QRQRPAEIK), and CDR2 40-61 were synthesized by use of standard solid-phase methods, purified by high-performance liquid chromatography, and identified by amino acid sequence analysis.[15]

Antibodies

Jovi 1.3 cells, which secrete a murine monoclonal antibody specific for human TcR-Vβ3,[16] were a kind gift of Dr. J. Viney (Imperial Cancer Research Fund, London, U.K.). Antibodies for cell staining and population analysis were all purchased from Becton-Dickinson (Oxford, U.K.).

T-cell proliferation assays: Clonal

The T-cell clones used in these experiments were isolated as previously described.[17] HA1.7 was reactive to HA 307-319 peptide and expressed the Vβ3 TcR, and the two HDM-reactive (responsive to both *D. pteronyssinus* and *D. farinae*) clones DE26 and DE9 were TcR-Vβ3 and TcR-Vβ6.7a, respectively. For proliferation assays the cells were cultured at 2×10^4 per well in round-bottom 96-well microtiter plates in complete RPMI-1640 medium (Gibco, Paisley, Scotland) supplemented with 2 mmol/L L-glutamine, 100 U/ml penicillin/streptomycin and 5% screened, heat-inactivated, A+ human serum (North London Blood Transfusion Service). An equal number of autologous Epstein-Barr virus–transformed B cells (5000 rad) or peripheral blood mononuclear cells (PBMCs) (2500 rad) of the correct haplotype were used as antigen-presenting cells. HA 307-319 or *D. farinae* and CDR2 40-61 were added at the concentrations indicated to the apppropriate wells to produce a final volume of 200 μl. As a control for toxicity, cells were incubated with CDR2 40-61 and 10% interleukin-2 (IL-2) (Lymphocult-T; Biotest Folex, Frankfurt, Germany) or in wells coated with anti-CD3 (10 μg/ml for 30 minutes at 37° C) and IL-2. After 72 hours of incubation, tritiated methylthymidine (1 μCi/well; Amersham International Inc., Amersham, U.K.) was added to cultures, which were harvested onto glass fiber filters 8 to 16 hours later. Proliferation was measured as tritiated methylthymidine incorporation per culture by means of liquid scintillation spectroscopy with the results expressed as mean counts per minute for 3 to 9 replicate wells. Standard deviation rarely exceeds 20%.

T-cell proliferation assays: Polyclonal

PBMCs from nonatopic and atopic donors were separated by discontinuous density centrifugation over Ficoll-Hypaque (Pharmacia Ltd., Milton Keynes, U.K.). PBMCs (1×10^5) were incubated in 96-well round-bottom microtiter plates with various combinations of HDM antigen (*D. pteronyssinus* extract) and CDR2 or K1-9 peptides at the concentrations indicated. PBMCs were also cultured with 10% IL-2, on plates coated with anti-CD3 antibody and IL-2, with the T-cell mitogen phytohemaggiutinin protein (10 μg/ml; Sigma Chemical Co., St. Louis, Mo.), MTSE, or the bacterial superantigen *Staphylococcus aureus* enterotoxin B (at 0.01 to 1 μg/ml, Sigma) in the presence or absence of CDR2 peptide. Complete RPMI-1640 medium was used at a final volume of 200 μl. After 6 days, cells were pulsed with 1 μCi/well of tritiated methylthymidine for 8 to 16 hours, after which the cells were harvested and proliferation measured as described above for T-cell clones.

Vβ3+, CD4+, and CD8+ T-cell depletions

PBMCs were depleted of Vβ3+, CD4+, or CD8+ T cells, respectively by immunomagnetic separation with Dynabeads (Dynal, New Ferry, U.K.) used according to the manufacturer's instructions. Briefly, to deplete PBMCs of Vβ3+ cells, they were incubated with Jovi 1.3 culture supernatant on ice for 30 minutes. Cells were then washed in the cold, and sheep anti-mouse Ig-conjugated magnetic beads were added at the approximate ratio of 10:1 beads to Vβ3+ T cells. The beads were washed three times before use to ensure complete removal of the azide in which the beads are stored. The mixture was rotated in the cold for an additional 30 minutes, and then Vβ3+ T cells were removed by a magnet. PBMCs were depleted of more than 90% CD4+ or CD8+ T cells in a similar fashion with commercially available Dynabeads coated with monoclonal antibodies binding the CD4 or CD8 antigens on human T cells. For CD8 depletion, two rounds of magnetic depletion were performed to optimize elimination of contaminating CD8+ T cells. The cells were analyzed by flow cytometry (Coulter EPICS Profile II; Coulter Corp., Hialeah, Fla.) to ensure purity.

RESULTS

Inhibition of polyclonal T-cell responses to HDM by Vβ3-CDR2 peptide

A peptide derived from the CDR2 region of the TcR-Vβ3 has been tested for its capacity to selectively inhibit the response of Vβ3+, HDM-reactive human T cells. PBMCs from an

atopic individual were stimulated with HDM antigen, *D. pteronyssinus* extract over a concentration range of 5 to 20 μg/ml in the presence or absence of CDR2 peptide, residues 40-61, derived from the human TcR-Vβ3 sequence. Addition of the Vβ3-CDR2 peptide resulted in a dose-dependent inhibition of polyclonal T-cell responses to *D. pteronyssinus* (Fig. 1, *A*). In a second donor substantial inhibition of the polyclonal response to HDM by the CDR2 peptide, but not by a control peptide derived from keratin (residues 1-9; Fig. 1, *B*), was observed. A second control peptide, overlapping the CDR2 region of a murine TcR-Vβ8 gene failed to inhibit the HDM response in five individuals: mean inhibition values in the presence of murine CDR2 TcR-Vβ8 and human CDR2 TcR-Vβ3 peptides were 4.4 ± 8.6 versus 52.0 ± 22.0, respectively.

The effect of the CDR2 peptide on peripheral T cells stimulated with either HDM or an irrelevant antigen, MTSE was also compared, and in contrast to HDM, the response to MTSE was not inhibited by the CDR2 peptide (Fig. 1, *C*). Furthermore, when investigated at concentrations of up to 300 μg/ml, the CDR2 peptide was not mitogenic and unable to inhibit T-cell proliferation stimulated by exogenous IL-2, by mitogens such as phytohemagglutinin protein, by cross-linked anti-CD3 antibody plus IL-2, or by the bacterial superantigen *S. aureus* enterotoxin B (data not shown).

Specificity of Vβ3-CDR2 peptide action

To demonstrate that CDR2 peptide inhibition of the HDM response is Vβ3-specific, the effects on Vβ3-depleted and unfractionated PBMCs were compared. After greater than 90% depletion of Vβ3-expressing T cells from PBMCs with a Vβ3-specific mononclonal antibody, a vigorous response to antigen was still observed, presumably reflecting the contribution of other TcR-Vβ regions, including Vβ6.7a and Vβ21, to the HDM response.[3] However, after Vβ3+ T-cell depletion, the HDM response was completely refractory to CDR2 peptide inhibition (Fig. 2, *A* and *B*). These experiments confirm that inhibition by TcR-Vβ3 CDR2 peptide of the polyclonal human T-cell response to HDM is dependent on the presence of Vβ3+ T cells.

Population study

Because Vβ3-CDR2 peptide inhibition of the polyclonal response to HDM appears to be spe-

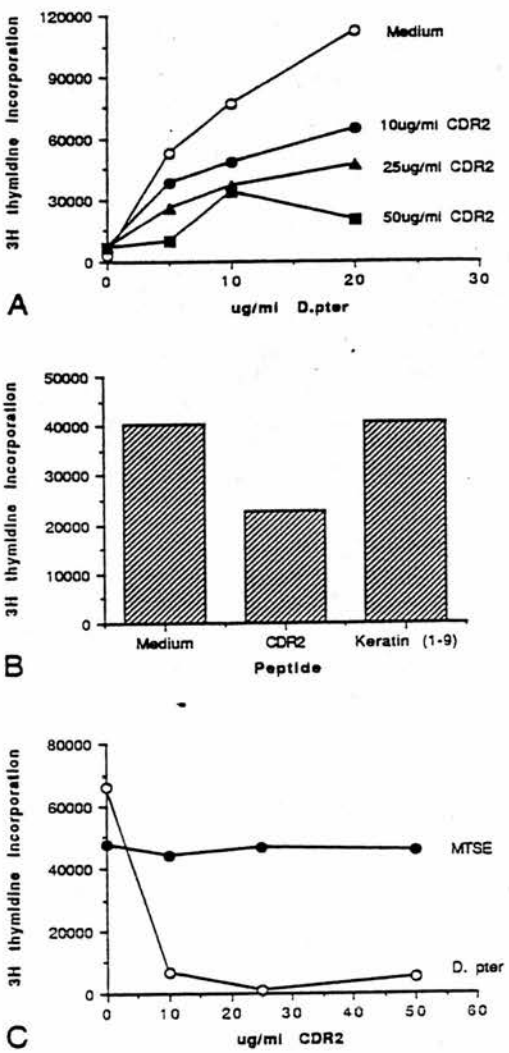

FIG. 1. Modulation of antigen-dependent polyclonal human T-cell responses by a synthetic peptide corresponding to the CDR2 region of the human TcR-Vβ3. PBMCs (1×10^6/well) were stimulated in 96-well microtiter plates with (**A**) 5 to 20 μg/ml HDM allergen *(D. pteronyssinus)* alone *(open circles)* or with 10 to 50 μg/ml CDR2 peptide *(filled symbols)*, (**B**) 20 μg/ml HDM in medium or together with 25 μg/ml CDR2 or keratin (K1-9) peptides, or (**C**) μg/ml HDM or MTSE control antigen in medium or with 20 CDR2 peptide. The experiments shown in **panels** A to C were performed with PBMCs derived from three separate donors. T-cell responses were assessed as tritiated methylthymidine uptake as described in the Methods section. Data are presented as mean tritiated methylthymidine incorporation from replicate (3 to 9) wells with a standard deviation of less than 20%.

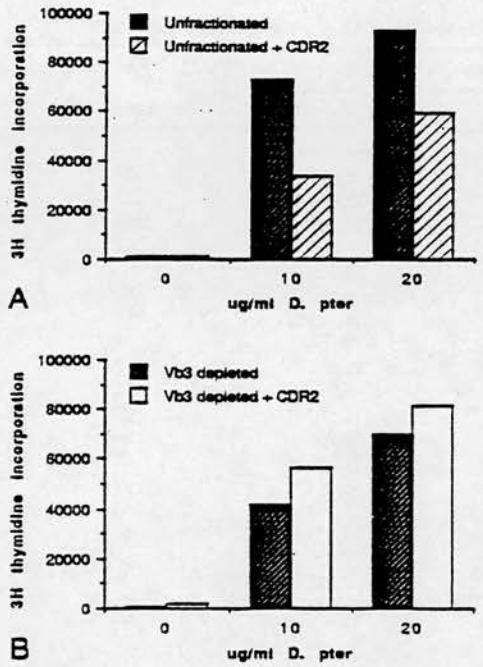

FIG. 2. Vβ3+ T-cell depletion leads to loss of CDR2 inhibition of the HDM response. Unfractionated (**A**) or Vβ3-depleted (**B**) PBMCs were cultured with HDM in the presence or absence of 25 μg/ml CDR2 peptide as described in the legend to Fig. 1 and in the Methods section.

cific, we have used this experimental approach to determine what percentage of HDM-reactive individuals have HDM responses inhibited by this peptide. A population of HDM-reactive individuals who were first seen with a wide range of clinical symptoms, ranging from mild atopy to severe asthma, was selected at random from the allergy clinic. These individuals were unrelated and assumed to show no skewing or bias in HLA or other genetic haplotypes. Included in this study were five unrelated, nonatopic individuals who showed in vitro responsiveness to HDM. The earlier data obtained in studies with a single individual suggested that TcR-Vβ3–expressing cells may be dominant in the HDM-reactive T-cell repertoire,[3] and this study of 21 donors suggests that this may be true within the population as a whole. Of 16 atopic donors tested, only two failed to show substantial inhibition of the HDM response by the CDR2 peptide. The magnitude of modulation varied considerably between donors (range 9% to 92%), presumably reflecting differences in the relative contribution of TcR-Vβ3 to the HDM response between individuals. It seems unlikely that when very high inhibition (>90%) of the HDM response occurs, the T-cell response of that individual is solely represented by Vβ3-expressing T cells. Rather, it seems probable that regulatory mechanisms induced by the CDR2 peptide in culture may have bystander effects on the polyclonal response to allergen.

There was no marked difference in the ability of the CDR2 peptide to inhibit the HDM response of either atopic (mean inhibition, 53.4% ± 25.0%) or nonatopic individuals (mean inhibition, 59.6% ± 9.3%) (Table I). Therefore although the same antigen appears to activate peripheral T cells from both atopic and nonatopic individuals, the functional consequences of T-cell activation differ between the two groups.[18]

Effect of CDR2 peptide on the response of HDM-specific T-cell clones

Two CD4+ Vβ3+ human T-cell clones, one reactive with a peptide derived from influenza virus hemagglutinin (HA 307-319), the other with HDM, and a third Vβ6.7a+, HDM-reactive T-cell clone were all resistant to CDR2 peptide–induced inhibition (Table I). This finding suggests that the modulatory effect of the TcR peptide may not simply be explained by a direct effect on the CD4+ Vβ3+ antigen-responsive T-cell population.

Role of CD8+ T cells

In animal studies it has been proposed that CDR2 inhibition of antigen-induced EAE acts through the induction of a CD8+ regulatory T-cell population.[3] To assess evidence for a similar regulatory population in human beings, the capacity of CDR2 peptide to inhibit the HDM response in unfractionated or CD8+-depleted human PBMCs was compared. With the use of antibody-conjugated magnetic beads, depletion of CD4 or CD8 populations by more than 90% was routinely achieved. The response to antigen was only observed in the CD4+ T cell–enriched population (Fig. 3, *A*). Nevertheless, after depletion of the CD8+ cells, the capacity to modulate the response by TcR peptide was lost (Fig. 3, *B* and *C*). CD8+ T-cell depletion resulted not only in loss of inhibition of the HDM response by the CDR2 peptide, but led to an increased response in the presence of the peptide. A similar increase was also observed after depletion of Vβ3+ T cells from PBMCs (Fig. 2). The reason for this is as yet

TABLE I. Inhibition of HDM allergen–dependent T-cell responses of HDM-allergic individuals

	Tritiated thymidine incorporation (cpm)			
	Medium	*D. pter*	*D. pter* + CDR2	Inhibition (%)
Donor				
A1	3,004	14,568	6,681	54
A2	1,031	32,216	21,454	33
A3	428	37,208	20,265	46
A4	3,037	112,067	20,306	82
A5	324	25,400	7,956	69
A6	251	13,589	4,320	68
A7	486	27,886	24,165	13
A8	265	22,294	7,784	65
A9	881	63,035	51,201	9
A10	5,988	16,246	7,993	51
A11	679	23,363	7,610	67
A12	1,327	73,281	33,716	54
A13	634	22,117	13,241	41
A14	397	66,297	5,162	92
A15	947	45,351	15,964	65
A16	3,798	27,538	9,359	66
NA1	356	5,575	1,321	76
NA2	467	71,003	32,354	55
NA3	759	15,629	6,731	57
NA4	378	26,703	12,483	53
NA5	4,744	32,415	13,763	57
T-cell clones				
HA1.7 (Vβ 3; HA)	70	12,800	13,283	0
DE26 (Vβ 3; HDM)	2,954	15,365	15,365	0
DE9 (Vβ 6.7a; HDM)	2,275	27,788	27,179	2

PBMCs from 16 atopic (A1-16) and five nonatopic (NA1-5) donors were cultured as indicated in Fig. 1 in medium alone or with 20 μg/ml HDM in the presence or absence of CDR2 peptide (data shown are for the concentration of CDR2 producing maximal inhibition of the HDM response for each donor: A2 10 μg/ml, the rest 25 or 50 μg/ml). T-cell clones (HA 1.7, DE26 and DE9) were cultured at 2×10^4 cells/well in the presence of an equal number of irradiated (2500 rad) histocompatible PBMCs in medium, 1 μg/ml HA 307-319, or 20 μg/ml *D. farinae*, respectively in the presence or absence of 25 μg/ml CDR2 peptide; and proliferation was measured on day 3 as described in the Methods section.

D. pter, *D. pteronyssinus*.

unclear, but it might reflect the presence of a subpopulation of CD4+ T cells that are also CDR2-reactive (but non inhibitory). It seems likely that, as in the animal studies, a CD8+ T-cell regulatory subset is induced by the CDR2 peptide that modulates the allergen responsive CD4+ T-cell population.

DISCUSSION

In this report we demonstrate that a TcR peptide, based on the sequence of the CDR2 region of the TcR-Vβ3 gene segment, downregulates the human polyclonal T-cell response to HDM. This effect is specific because it is dependent on the presence of Vβ3+ cells in the stimulation culture. In an earlier study we demonstrated, using anchor polymerase chain reaction analysis, that T-cell clones specific for HDM, derived from a single atopic individual, showed a bias towards TcR-Vβ3 usage. In this study inhibition of the polyclonal response to HDM by CDR2 TcR-Vβ3 peptide in the majority (16 of 21) of donors examined has been used to demonstrate that a bias toward Vβ3 usage occurs not only in clones but, more relevantly, among the polyclonal responses of peripheral blood derived, HDM-responsive T cells within the population as a whole. Although T cells expressing TcR other than Vβ3 clearly contribute to the overall HDM-reactive repertoire,[3] our results demonstrate that Vβ3+ T cells are a major

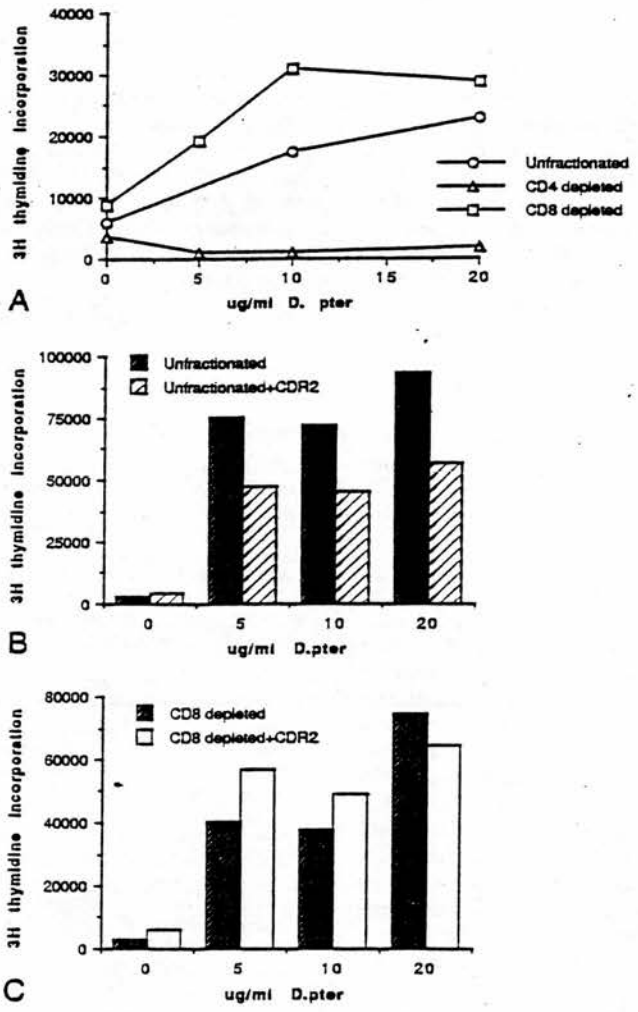

FIG. 3. Effect of CD8+ cell depletion on CDR2 modulation of the HDM response. A, PMBCs were depleted of either CD4+ or CD8+ cells with specific antibody directly conjugated to magnetic beads, and the proliferative response to HDM of unfractionated, CD4-depleted, or CD8-depleted PBMCs was compared. B, Unfractionated or (C) CD8-depleted PBMCs were cultured with HDM and 25 μg/ml CDR2 peptide.

component of the HDM-reactive repertoire. Although the relative contribution of Vβ3+ T cells to the HDM response will vary between individuals, because inhibition of up to 90% is achievable, it is possible that once the regulatory mechanism has been activated, nonspecific bystander mechanisms may operate to dampen the polyclonal response to antigen.[19-21] An example of this has been described in the model of murine responses to the group I allergen of *D. pteronyssinus* in which intranasal administration of an immunodominant peptide was able to tolerize mice to subsequent challenge with the intact protein.[21]

The goal in the treatment of immunologically mediated diseases has been to design therapies that specifically target T lymphocytes that induce

or regulate the disease process and to avoid nonspecific immunosuppressive regimens and the long-term problems associated with their use. To design such therapies, it is necessary to define the dominant T-cell epitope, the presence of limited TcR usage or HLA restriction involved in the disease inducing immune response, which can be specifically targeted. The design of this study demonstrates that it is possible to use peptide analogs of the appropriate TcR-Vβ region to specifically target and significantly inhibit the proliferative response of allergen-reactive T cells. Although these studies have as yet only demonstrated inhibition of the human T-cell response to allergen in vitro, it is noteworthy that similar studies have been performed in vivo in both rat and mouse models of EAE, in which restricted Vβ8.2 usage by encephalitogenic T cells was observed. In these studies the appropriate CDR2 peptides not only prevented the onset of clinical disease but also, more practically, caused regression of ongoing EAE.[12]

Initial studies to address the mechanism whereby CDR2 peptides modulate the polyclonal response to allergen suggest that induction of a CD8+ regulatory T-cell subset by the peptide, which modulates the responsiveness of HDM-responsive T cells, may occur. In experiments with T-cell clones we find no evidence for a direct effect of the peptide on HDM-reactive T cells, which suggests that the CDR2 peptide is not directly binding the TcR, nor is it competing with antigen at the level of MHC binding. Failure to block phytohemagglutinin or anti-CD3–induced T-cell proliferation suggests that the CDR2 peptide does not act through CD2 or the T cell–specific antigen receptor. More direct evidence comes from studies in which depletion of CD8+ T cells from the HDM stimulation cultures leads to loss of CDR2 peptide–mediated inhibition. The antigenic peptide recognized by the putative "regulatory" CD8+ T-cell subset, which is presumably derived from the sequence of TcR expressed on Vβ3+ HDM-reactive CD4+ T cells, and the mechanism or mechanisms by which the CD8+ T cells modulate the response of these cells remain to be clarified in future experiments.

TcR peptide–based therapy may have broader applications in allergic and autoimmune diseases in which TcR gene usage is reported to be restricted (e.g., multiple sclerosis and myasthenia gravis in which Vβ5.2 and Vβ12, respectively, are implicated).[22-24] In certain diseases, however, such as rheumatoid arthritis, the observed TcR expression of cells infiltrating disease sites appears to be patient-specific rather than disease-specific, and involves multiple Vβ families.[25-28] Nevertheless, specific therapy based on TcR structure may have a practical application in allergic disease. The inactivation of selected components of the specific T-cell repertoire, such as those T cells that express the dominant TcR-Vβ specificity and represent long-lived allergen reactive clones,[3] may be sufficient to downregulate the severity of the clinical phenotype.

We thank Drs. Brigitte Askonas and Richard Lake for critical comments on the manuscript, and Mr. John Hayball for the synthesis of peptides used in this study.

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Experimental Therapeutics (A)

International Archives of Allergy and Immunology

Int Arch Allergy Immunol 1999;118:122–124

Linked Suppression in Peripheral T Cell Tolerance to the House Dust Mite Derived Allergen Der p 1

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Key Words
Notch signalling · Peripheral tolerance · T cells · Der p 1 · Linked suppression

Abstract
Background: Peripheral tolerance is required to maintain balance within the immune system. A feature of peripheral tolerance is linked suppression, in which tolerance induced to a single T cell epitope inhibits the response to all epitopes in the same protein. It is suggested that this phenomenon is mediated by regulatory T cells through either the activity of immunopressive cytokines or direct cell contact. In previous experiments we failed to detect inhibitory cytokines when T cells from mice rendered tolerant by intranasal delivery of the immunodominant peptide of Der p 1 (p 1, 110–131) were restimulated with peptide in vitro. Therefore, the aim of this study was to determine if cognate interactions between T cells mediated by Notch/Delta signaling induce and maintain peripheral T cell tolerance. ***Methods:*** Using in situ hybridization and viral mediated gene transfer, the expression and function of Delta1 were investigated in a murine model of T cell tolerance to Der p 1 in vivo. ***Results:*** Delta1 expression is increased on peripheral T cells during the induction of tolerance with high-dose peptide delivered intranasally and when tolerant animals are rechallenged under immunogenic conditions. Peptide p 1, 110–131-specific CD4+ T cells transfected with Delta1 inhibited the response of antigen-primed T cells and induced linked suppression. ***Conclusions:*** High-dose peptide delivered intranasally induces transient expression of Delta 1 on inhibitory CD4+ T cells. Ligation of the Notch1 receptor on neighbouring T cells by Delta1+ regulatory T cells inhibits clonal expansion of the former and mediates linked suppression.

Introduction

Immune responsiveness to inhaled and ingested antigens that are non-pathogenic occurs through the induction of peripheral tolerance [e.g., 1]. Several studies have now been described in which the mechanisms underlying tolerance induction following the delivery of antigen via mucosal surfaces have been investigated [e.g., 1, 2–4]. We have demonstrated that intranasal administration of the immunodominant T cell epitope (residues 110–131) of the group 1 allergen of *Dermatophagoides pteronyssinus*, Der p 1, at high doses induces extreme and long-lasting tolerance [2, 5, 6]. Tolerance to the immunodominant epitope inhibited T cell responses to all other epitopes in Der p 1, provided that tolerant mice were challenged with the intact protein. Furthermore, this phenomenon, which is termed linked suppression, was mediated by CD4+ T cells [6]. Linked suppression has also been described in other models of

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peripheral tolerance to transplantation and self-antigens [7–9]. The processing and presentation of multiple epitopes originating from within the same protein (Der p 1) by a single or neighbouring antigen-presenting cell brings regulatory and naive T cells into close proximity and facilitates the transfer of inhibitory signals between the T cell populations. It has been proposed that the secretion of immunosuppressive cytokines, such as interleukins 4 and 10 and transforming growth factor beta, provides the negative signals that mediate the inhibitory effects observed in linked suppression [e.g., 1, 9]. However, we have been unable to detect either transforming growth factor beta or Th2-type cytokine production by T cells from tolerant mice when restimulated with peptide in vitro. Therefore, it is possible that linked suppression in our system uses an alternative mechanism which requires direct cell contact between the regulatory and naive T cells, similar to that reported for bystander suppression mediated by anergic human T cell clones in vitro [10, 11].

The Notch signalling pathway regulates the differentiation and growth of a range of tissues [e.g., 12], including the immune system, where in the thymus, for example, it appears to play a role in T cell development [e.g., 13]. In studies on neurogenesis, in which this pathway has been studied in most detail, it has been demonstrated that Notch signalling regulates cell differentiation through a cell contact dependent mechanism, termed lateral inhibition [14]. This mechanism involves the receptor Notch 1 binding to the ligand Delta1. This prompted us to investigate whether Notch1/Delta1 interactions between T cells contribute to peripheral tolerance and mediated linked suppression.

Materials and Methods

In brief, two experimental protocols have been used in the experiments reported here. Firstly, the expression of Delta1-specific transcripts, which are measured by in situ hyridization, in sections prepared from draining lymph nodes isolated from tolerant, immunized and unprimed H-2^b (C57BL/6J) mice at different time points. Tolerance is induced as previously described [2]. Briefly, peptide p 1, 110–131 (100 μg), dissolved in phosphate-buffered saline is administered intranasally over 3 consecutive days. The draining cervical lymph nodes and spleens are removed at selected times and T cell responses measured in vitro. To assess responses in the rechallenge phase of tolerance, peptide-pretreated and control mice are immunized with Der p 1 (50 μg) 2 weeks after treatment and responses measured in vitro [2]. The second protocol involves the adoptive transfer of p 1, 110–131-specific CD4+ T cells transfected with Delta1 into naive mice. The mice are then immunized with Der p 1 in adjuvant, and the proliferative response and cytokine production are measured for splenic and draining lymph node T cells restimulated with antigen in vitro.

Results and Discussion

Tolerance induced by peptide delivered via the respiratory mucosa resulted in a marked increase in the number of Delta1-expressing cells as determined by in situ hyridization. The expression of Delta1 was maximal at 2 days, but, although still detectable on day 8, had declined to approximately 50% of peak levels. In contrast, Delta1 transcripts in uprimed mice were barely detectable. Rechallenging tolerant mice with an immunogenic dose of Der p 1 in adjuvant resulted in Delta1 expression at levels higher than those observed following tolerance induction. However, increased expression of Delta1 was not seen following active immunization of non-tolerant (naive) mice with Der p 1 in adjuvant. These results demonstrate that Delta1 expression is transiently upregulated as a consequence of peptide-induced tolerance. Furthermore, Delta1 is re-expressed when tolerant mice are exposed to antigen rechallenge under conditions that normally prime, and, therefore, this suggests that Notch/Delta1 signaling may be important in the maintenance as well as in the induction of peripheral tolerance.

In order to further investigate the function of Notch/Delta1 signaling in T cell tolerance, the Delta1 gene was expressed in p 1, 110–131 reactive CD4+ T cells. The Delta1+ or control infected T cells were irradiated and injected into naive mice which were subsequently immunized with Der p 1 or ovalbumin in adjuvant. Lymph node T cells from the mice that had received the Delta1+ T cells and had been immunized with Der p 1 failed to proliferate when rechallenged with Der p 1 in vitro. In contrast, T cell responses in the mice that were primed with ovalbumin remained intact. These studies were extended, and the T cell recognition of minor (e.g., residues 81–102) as well as the dominant T cell epitope in Der p 1 was examined. We observed that the adoptive transfer of the Delta1+ T cells, reactive with p 1, 110–131, inhibited responses to both the dominant and minor determinants. The results of these experiments suggest that CD4+ T cells expressing Delta1 mediate similar effects to regulatory T cells selected by high doses of peptide in that they induce antigen-specific peripheral T cell tolerance and mediate linked suppression.

During the induction of tolerance, transient activation and expansion of T cells occurs initially [e.g., 5, 6] which suggests that tolerance does not occur as the result of defective T cell receptor or costimulatory receptor mediated signalling. However, since the final functional outcome is unresponsiveness, other negative signals must override costimulation which together with antigen recognition is required for productive immunity. Our results suggest that in peripheral tolerance Delta1 transiently expressed on in-

hibitory T cells may ligate Notch which is constitutively expressed on naive T cells. These T cell populations cluster at the membrane of the same antigen-presenting cell which presents both the major and minor T cell epitopes, allowing cell contact to occur and negative signals to be transmitted. Thus, the outcome of Notch signalling in this instance is to prevent clonal expansion and to mediate linked suppression.

Acknowledgements

This work was supported by the British Lung Foundation, the Wellcome Trust and the Medical Research Council.

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2.3 ANTIGEN MEDIATED MODULATION OF T CELL FUNCTION

2.3.5 SELECTIVE ANTIGEN PRESENTATION BY VACCINE VECTORS

Harris SJ et al: *Int Immunol* 1997, **9**:273-280.

Lamb JR: *Res Immunol* 1998, **149**:235-240

Hetzel C et al: *Infect Immun* 1998, **66**:3643-3648

International Immunology, Vol. 9, No. 2, pp. 273–280

Prediction of murine MHC class I epitopes in a major house dust mite allergen and induction of T1-type CD8$^+$ T cell responses

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Keywords: allergen presentation, allergen-specific CD8$^+$ T cells, T_h1/T1 T cell priming

Abstract

The group I (Der p 1) allergen of *Dermatophagoides pteronyssinus* (house dust mite, HDM) contains several T helper (T_h) epitopes recognized by C57BL/6 mice, with the peptide (111–139) containing a dominant MHC class II-restricted epitope (113–127). Since CD8$^+$ T cells are thought to play a role in the regulation of allergic disease, we examined the Der p 1 sequence for potential MHC class I-binding motifs and observed that residues 111–119 (FGISNYCQI) contain motifs for H-2D^b and K^b. Furthermore, immunization of C57BL/6 mice with unadjuvanted Ty virus-like particles (VLP) carrying Der p 1 (111–139), a method known to induce murine cytotoxic T lymphocyte (CTL) responses, primed Der p 1 (111–119)-specific D^b-restricted CTL which produce high levels of IFN-γ and low levels of IL-5 and IL-6 *in vitro* (T1-type CTL). VLP carrying the minimal epitope (FGISNYCQI) also induced a CTL response following immunization without adjuvant by various routes. Der p 1 (111–139)-VLP adjuvanted with alum did not prime CTL in C57BL/6 mice but were found to prime T_h1-type CD4$^+$ T cells that recognize the overlapping peptide (113–127) and native protein. The ability to successfully predict allergen-specific CD8$^+$ T cell epitopes and prime CD8$^+$ and/or CD4$^+$ T cell responses provides an opportunity to dissect the relative roles of these T cells in the regulation of allergic responses and may offer therapeutic potential for reprogramming T_h2-type allergic responses.

Introduction

The production of specific IgE antibodies predisposes individuals to become sensitized to environmental allergens and plays an important role in mediating the allergic inflammatory response. The synthesis of IgE by B cells is dependent on the cognate interactions of CD4$^+$ T cells of the T_h2-type producing IL-4, and other inflammatory cytokines IL-5 and IL-6 (1–3). Studies in rodents have demonstrated that following allergen inhalation, CD4$^+$ T_h2-type cells are activated and can promote a transient production of allergen-specific IgE (4,5). However, this antibody production is short lived and CD4$^+$ T_h2 cells are replaced in time by CD4$^+$ T_h1-type cells which block further expansion of T_h2 cells by secretion of cytokines such as IFN-γ (6,7). A similar series of events is thought to occur in normal human responses to respiratory and dietary antigens, whereas in the atopic individual IgE antibody levels persists, driven by a dominant T_h2-type CD4$^+$ T cell response.

House dust mite (HDM, *Dermatophagoides pteronyssinus*) allergy represents a significant problem in the population and ~10% of people suffer with allergic symptoms following exposure to house dust (8). Although both non-atopic and atopic individuals produce immune responses to HDM allergens, the atopic patients display a T_h2-dominant phenotype (9). There are several possible therapeutic approaches to down-regulate allergic T cell responses *in vivo*. For example, it is possible to functionally inactivate allergen-specific T_h cells by the tolerogenic administration of allergen-derived peptides (10–13). Alternatively, immune deviation may be provoked by shifting the balance of cytokine production from T_h2- to T_h1-type and this may be accomplished by priming T_h1-type cells (14), by inhibiting T_h2 cytokines using mAb (15) or by providing T_h1-type cytokines (7,16).

A potentially novel approach would be to activate allergen-specific CD8$^+$ T cells. The benefit of such an approach is supported by rodent studies which have shown that CD8$^+$

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Received 15 July 1996, *accepted* 28 October 1996

T cells become activated following allergen exposure, and can down-regulate the IgE response and normalize airway responsiveness in sensitized animals (17,18). Furthermore, recent studies have shown that allergen-specific $CD8^+$ T cells can be induced following injection of mice with an allergen-based naked DNA vaccine (19). Although $CD8^+$ T cells have been studied in various animal models of allergy, a common deficit of all the studies has been the failure to characterize the allergen epitope specificity of the $CD8^+$ T cells.

In this report we describe the identification of a $CD8^+$ T cell epitope derived from the HDM allergen Der p 1, which is a major target antigen for the allergic immune response in humans (20). The sequence 111–119 of Der p 1 was identified as a possible D^b- or K^b-restricted epitope based on putative MHC class I-binding motifs (21,22). The region (111–139) is of significance in that it represents an immunodominant region which is recognized by $CD4^+$ T cells in both humans (13) and C56BL/6 mice (12). Furthermore, intranasal immunization with Der p 1 (111–139) peptide tolerized mice to subsequent challenge with the native Der p 1 protein (11).

To characterize responses to this region we produced recombinant hybrid Ty virus-like particles (VLP), an antigen presentation system known to induce strong cell-mediated immune responses (23,24,25), carrying the sequences 111–139 and 111–119 of Der p 1. We show that both of these constructions, when injected without adjuvant, prime classical H-$2D^b$-restricted cytotoxic T lymphocytes (CTL) in C57BL/6 mice which kill target cells pulsed with peptide 111–119. In addition, the CTL secreted Tl-type cytokines when activated *in vitro* and could be induced when the VLP were administered through different routes. When injected with alum adjuvant, the Der p 1 (111–139)-VLP primed T_h1-type proliferative T cells against the 113–127 MHC class II-restricted epitope but did not prime CTL to the overlapping epitope.

Methods

Immunization of mice

Female C57BL/6 mice, 6–8 weeks old, were obtained from Charles River (Margate, UK). Mice were immunized by the subcutaneous route (s.c.) at the base of the tail for investigation of proliferative T cell responses in draining lymph nodes (DLN) and the intramuscular (i.m.), intraperitoneal (i.p.) or intranasal (i.n.) routes were used for the generation of CTL responses in the spleen.

Preparation of VLP and peptides

Hybrid Ty-VLP were constructed and purified as previously described (26,27). Briefly, TYA:Der p 1 fusion genes were constructed by inserting synthetic oligomers encoding the Der p 1 sequences (22) into a yeast expression vector (pOGS40) that contains a truncated TYA gene encoding amino acids 1–381 of protein p 1. The resulting plasmids produce self-assembling hybrid Der p 1-VLP. These were purified by sucrose density gradient followed by size exclusion chromatography. The T_h epitope in 111–139 is predicted to be 113–127 based on the known I-A^b motif (28). Peptides were dissolved in 5% DMSO and PBS, and stored at –70°C in aliquots. Native Der p 1 protein was extracted and purified from spent HDM medium, as previously described (11,12).

The following VLP were produced:

Der p 1 (111–139)-VLP	FGISNYCQIYPPNANKIREA-LAQPQRYCR
Der p 1 (111–119)-VLP	FGISNYCQI
Der p 1 (113–127)-VLP	ISNYCQIYPPNANKI

The following synthetic peptides were made by Genosys (Cambridge, UK)

Der p 1 (113–127)	T helper (I-A^b) epitope	ISNYCQIYPPNANKI
IgGVH (59–74)	control (I-A^b) epitope (21)	HNADFKTPATLTVDK
Der p 1 (111–119)	CTL (H-2^b) epitope	FGISNYCQI
VSV NP (53–60)	CTL (H-2^b) epitope	RGYVYQGL
INP (366–374)	CTL(H-2^b) epitope	ASNENMETM

Proliferation and cytokine assays

The methods were as previously described (23). Inguinal DLN nodes were removed 7 days after immunization and expressed through 100 μm mesh (Falcon 2350; Becton Dickinson, Franklin Lakes, NJ). The cells were cultured at 5×10^5/100 μl of RPMI 1640 supplemented with 5% FCS, L-glutamine and antibiotics in U-well microtitre plates. Stimulating peptides and proteins were added in a further 100 μl. The cells were cultured for 5 days and 0.5 μCi [^{3}H]thymidine/well was added during the final 4 h. The cells were harvested onto glass fibre and counted in a Wallac Microbeta scintillation counter. Results are expressed as stimulation indices (SI), where SI = c.p.m. in stimulated cultures/c.p.m. in control cultures. Supernatants for cytokine assays were obtained by culturing 10^7 lymph node or spleen cells in 2 ml wells with the appropriate peptide for 48 h. Supernatants were stored at –70°C prior to assay. IL-5, IL-6 and IFN-γ assays were performed using murine ELISA kits (Endogen, Boston, MA) according to the manufacturers instructions. Each culture supernatant and standard was assayed in duplicate. The results are expressed in pg/ml.

Cytotoxicity assay as a functional readout for CD8⁺ T cells

We have previously shown that the CTL primed by hybrid Ty-VLP are $CD8^+$ and MHC class I-restricted, and CTL assays were performed as described previously (24,25). Splenocytes were prepared from two mice per group. The pooled cells were cultured in 10 ml of RPMI-1640 medium supplemented with 10% FCS, 5×10^{-5}M 2-mercaptoethanol, L-glutamine, antibiotics and 10 μg Der p 1 (111–119) peptide. After 6–7 days, the effector cells were incubated for 4–5 h with Der p 1 (111–119) peptide-pulsed or control ^{51}Cr-labelled EL-4 target cells at effector:target ratios as shown. The ^{51}Cr released into the supernatant was measured in a Wallac Microbeta scintillation counter. The percent specific lysis was calculated as 100×[(test counts – mean spontaneous count)/(mean maximum counts – mean spontaneous counts)]. Results are expressed as net percent specific lysis (peptide-pulsed target

percent specific lysis – control target percent specific lysis). Figures show the mean net percent specific lysis of quadruplicate wells and standard error of the mean. In some experiments, purified CD8⁺ T cells were removed from splenocytes using an anti-CD8⁺ affinity column (Minimacs: Miltenyi Biotechnology, Surrey, UK). The levels of CD8⁺ and CD4⁺ T cells in the various cell preparations was evaluated by FACS analysis (FACScan; Becton Dickinson, Oxford, UK).

Results

Prediction of CD8⁺ epitopes in Der p 1 and generation of CTL responses with Der p 1-VLP

Given the putative role for CD8⁺ T cells in the down-regulation of allergic disease (17,18,29,30), we surveyed the Der p 1 sequence for known MHC class I-binding motifs (21,22) and identified 11 putative epitopes, five of which were predicted to bind to either K^b or D^b molecules. One of these, the Der p 1 sequence 111–119 (FGISNYCQI) conformed to the motif described for both K^b and D^b molecules (D^b motif, N at position 5 and I at position 9; K^b motif, Y at position 5 and I at position 8) and this sequence is in the previously identified immunodominant region Der p 1 (111–139). Since we had previously shown that hybrid VLP were potent inducers of CD8⁺ T cell responses when injected into mice without any adjuvant (24,25) we immunized C57BL/6 mice i.m. with 100, 20 or 4 μg Der p 1 (111–139)-VLP without adjuvant to look for CTL priming to the predicted epitope. Splenocytes were used as a source of primed CTL, because previous studies have shown that CTL leave the DLN and accumulate in the spleen within 7 days of priming with hybrid VLP (S. J. Harris and G. T. Layton, unpublished observations). Splenocytes were prepared on day 19 and re-stimulated with 1 μg/ml of Der p 1 (111–119) peptide. The effectors were tested against Der p 1 (111–119) peptide-pulsed and control EL-4 target cells after 6 days. Strong lysis of peptide-pulsed target cells was observed with effector cells from mice receiving 100 and 20 μg dose (Fig. 1). However, the response with the 4 μg dose, representing 0.1 μg Der p 1 epitope, was much lower. The predicted epitope, therefore, generates a H-2^b-specific CTL response when presented on hybrid VLP. The CTL epitope (111–119) is overlapping but distinct from the CD4⁺ T helper epitope (113–127) and does not induce proliferation in draining lymph node cells from mice immunized with Der p 1 (111–139)-VLP with or without alum adjuvant (data not shown).

Specificity and MHC restriction of CD8⁺ CTL responses

Because the CTL epitope has both the K^b and D^b motifs, we performed cold peptide inhibition of target cell lysis using peptides known to bind to either K^b or D^b. In order to establish the optimal and sub-optimal concentrations of Der p 1 (111–119) peptide for labelling target cells, various concentrations of peptide were added directly to a mixture of ^{51}Cr-labelled EL-4 targets and Der p 1-specific CTL effector cells from mice immunized with 100 μg Der p 1 (111–139)-VLP (E:T ratio 50:1). The concentration of peptide became limiting at 1 ng/ml, and was insufficient to label target cells at 0.1 ng/ml (Fig. 2A). This demonstrated the requirement for Der p 1

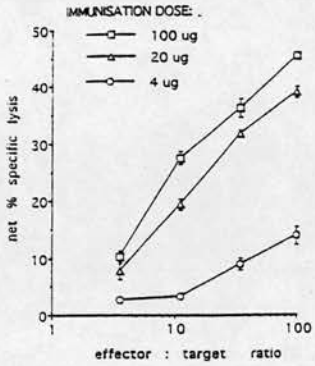

Fig. 1. Induction of CD8⁺ Der p I-specific CTL in C57BL/6 mice. Mice were immunized with 100, 20 or 4 μg Der p 1 (111–139)-VLP. Spleen cells were removed after 7 days and re-stimulated *in vitro* for 6 days with Der p 1 (111–119) peptide prior to testing against Der p 1 (111–119) peptide-pulsed or unpulsed target cells. Non-specific killing of unpulsed target cells at an E:T ratio of 100:1 was 12.0% (100 μg dose), 17.0% (20 μg dose) and 17.6% (4 μg dose).

peptide in target cell killing. We then tested the ability of known D^b [INP (366–374)] and K^b [VSV NP (53–60)] binding peptides to inhibit the binding of Der p 1 (111–119) to target cells. Der p 1-specific CTL effector cells, generated as above, and ^{51}Cr-labelled EL-4 target cells were plated at 50:1 cell ratio to which was added Der p 1 (111–119) at 1 ng/ml and either VSV NP (53–60) or INP (366–374) peptides at 100, 10, 1 or 0 ng/ml. The D^b-binding sequence INP (366–374) inhibited the lysis of target cells when present at 100 ng/ml and showed some inhibition at 10 ng/ml (Fig. 2B). However, the K^b-binding sequence showed no effect. This confirms the MHC class I-restricted nature of the killing and indicates that INP (366–374) and Der p 1 (111–119) are competing for the D^b molecule. The Der p 1 epitope is, therefore, D^b-restricted.

Cytokine production by Der p 1-specific CD8⁺ CTL

To further characterize the Der p 1-specific CD8⁺ T cell response, the production of IFN-γ, IL-5 and IL-6 by splenocytes from primed mice following Der p 1 (111–119) re-stimulation *in vitro* was investigated. C57BL/6 mice were immunized with 50 μg Der p 1 (111–139)-VLP with or without alum. Splenocytes were prepared on day 6 and re-stimulated with the Der p 1 (111–119) peptide *in vitro*. After 48 h, the supernatants were removed and assayed for cytokines as described above. In separate cultures, following 6 days re-stimulation, effector cells were tested for CTL activity. The results are shown in Table 1. Only splenocytes from mice immunized with Der p 1 (111–139)-VLP without alum adjuvant showed significant CTL, as previously established (27). CTL activity was associated with very high levels of IFN-γ but low levels of IL-5 and IL-6. CTL responses were not detected in the DLN or spleens of mice immunized with adjuvanted VLP.

Having defined the D^b-restricted epitope as Der p 1 (111–119) (FGISNYCQI) we investigated the ability of VLP carrying only this sequence to induce a Der p I-specific CTL response.

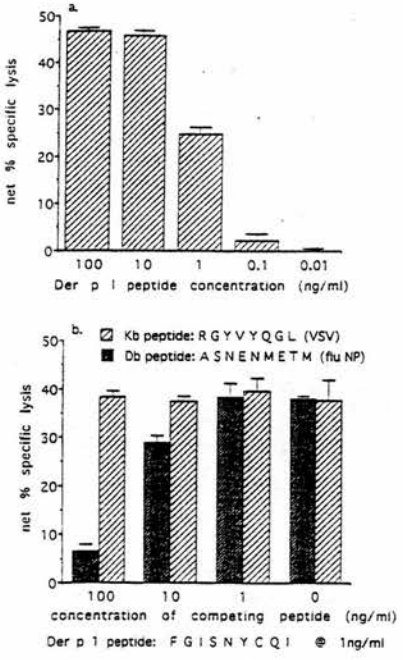

Fig. 2. (A) Dose response of Der p 1 (111–119) peptide for labelling EL-4 target cells in Der p 1 CTL assay. Effector cells from C57BL/6 mice immunized with Der p 1 (111–139)-VLP and Der p 1 (111-119) peptide were added to target cells to give an E:T ratio of 50:1 and peptide concentrations of 0.01–100 ng/ml. Non-specific killing of unpulsed target cells was 12.0%. (B) Ability of D^b-specific flu NP(366–374) peptide to compete with Der p 1 (111–119) peptide for binding to the D^b molecule as observed in a Der p I-specific CTL assay. Der p 1-specific effector cells were added to target cells at a ratio of 50:1. Der p 1 (111–119) peptide was present at 1 ng/ml and K^b-specific VSV(53–60) or D^b-specific INP(366–374) peptides were added at 0–100 ng/ml.

Potent CTL responses were primed using 100, 20 and 4 μg doses (all >35% net specific lysis at an E:T ratio of 33:1) and these VLP were five to 10 times more potent than Der p 1 (111–139)-VLP. These splenocyte effector cells also produced high levels of IFN-γ. In order to confirm that it is the $CD8^+$ T cells within the spleen cell population which produce the IFN-γ, $CD8^+$ T cells were isolated from the spleens of Der p 1 (111–119)-VLP-immunized (50 μg, i.m.) and control mice by affinity purification. The cells were ~90% $CD8^+$ T cells with 4% contaminating $CD4^+$ T cells by FACS analysis. These cells (2×10^6) were added to Der p 1 (111–119) peptide-pulsed normal splenocytes (1×10^7) in 2 ml wells and the supernatants removed after 48 h. Unfractionated splenocytes and the $CD8^-$ fraction from Der p 1 (111–119)-VLP-immunized and control mice were also re-stimulated with the peptide for 48 h. The results shown in Table 2 confirm that only cultures containing $CD8^+$ T cells from immunized mice produced IFN-γ. Immunized splenocytes depleted of $CD8^+$ T cells produced 24-fold less IFN-γ (520 pg/ml) compared to unfractionated splenocytes (12,400 pg/ml). This IFN-γ was presumably produced by the residual 3% $CD8^+$ T cells detected by FACS analysis. The much higher levels of IFN-γ produced in this experiment compared to those in Table 1 also suggests an increase in potency of Der p 1 (111–119)-VLP over Der p 1 (111–139)-VLP in terms of CTL induction.

Effects of route of administration on CTL induction by VLP carrying the minimal nine amino acid epitope

C57BL/6 mice were immunized with 50 μg Der p 1 (111–119)-VLP in PBS by either the i.m, i.p. or i.n. routes. Mice were anaesthetized for i.n. immunization. Five days following immunization, splenocytes were re-stimulated with Der p 1 (111–119) peptide *in vitro* for 6 days. A strong Der p 1-specific CTL response was seen in all groups (Fig. 3). Twelve weeks after immunization, a response was still present in mice immunized by the i.m. and i.p. routes, but was greatly diminished in the splenocytes from i.n. immunized mice (Fig. 3).

Induction of Der p 1-specific $CD4^+$ T_h1-type T cell responses using Der p 1 (111–139)-VLP

Since the Der p 1 (111–119) sequence overlaps the previously identified I-A^b-restricted epitope (113–127), we investigated

Table 1. Cytotoxic T cell (CTL) and cytokine responses by splenocytes 6 days following i.m. immunization with 50 μg Der p 1 (111–139)-VLP with or without alum adjuvant

In vitro	*In vivo*			
	Der p 1 (111–139)-VLP in alum		Der p 1 (111–139)-VLP no adjuvant	
	p (111–119)[a]	Control[b]	p (111–119)[a]	Control[b]
CTL (66:1)	0.9%	not tested	34.4%	not tested
IFN-γ (pg/ml)	<47	<47	5658.6	<47
IL-5 (pg/ml)	23.5	15.6	26.4	26.2
IL-6 (pg/ml)	139.6	87.0	373.4	137.7

[a]Splenocytes re-stimulated with peptide (111–119) from Der p 1 for 48 h (cytokines) or 6 days (CTL).
[b]Control wells were unstimulated splenocytes.

Table 2. IFN-γ production by purified CD8⁺, CD8⁻ and unfractionated splenocytes from Der p 1 (111–119)-VLP-immunized (50 μg, i.m.) mice re-stimulated *in vitro* with Der p 1 (111–119) peptide (10 μg/ml)

Cells[a]	Cells/well	IFN-γ (pg/ml)
Immune splenocytes	10^7	12,400
Control splenocytes	10^7	<47
Immune CD8⁺ cells plus peptide-pulsed control splenocytes	2×10^6 10^7	17,600
Control CD8⁺ cells plus peptide-pulsed control splenocytes	2×10^6 10^7	<47
Immune CD8⁻ splenocytes	10^7	520
Control CD8⁻ splenocytes	10^7	<47

[a]Splenocytes removed 7 days after priming and cells re-stimulated for 48 h (IFN-γ).

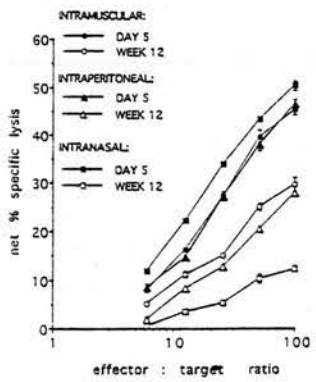

Fig. 3. Induction of Der p 1 (111–119)-specific CTL responses following immunization of C57BL/6 mice with 50 μg Der p 1 (111–119)-VLP by different routes. The generation of Der p 1 (111–119)-specific CTL responses was investigated at day 5 and week 12 following immunization by re-stimulating splenocytes for 6 days *in vitro* with Der p 1 (111–119) peptide and testing effectors against peptide-pulsed target cells. Non-specific killing of unpulsed target cells at an E:T ratio of 100:1 was <5.0% in all groups.

the ability of Der p 1 (111–139)-VLP to prime CD4⁻ T cells recognizing this epitope. Seven days following s.c. immunization of C57BL/6 mice with 60 μg Der p 1 (111–139)-VLP prepared in 300 μg alum, marked proliferative responses were observed to Der p 1 (113–127) peptide at 1 μg/ml and the native Der p 1 protein at 10 μg/ml in DLN cells (Fig. 4). The specificity of the response was corroborated by the lack of response to the control peptide and in mice immunized with alum alone. Strong proliferation was also observed to the Ty protein, as previously shown (23).

In order to characterize the proliferative T cell response further, 10^7 DLN cells from mice immunized with 50 μg Der p 1 (111–139)-VLP with or without alum were cultured for 48 h in 2 ml wells and the cytokine responses to Der p 1 (113–127) measured. Proliferative T cell responses in these DLN

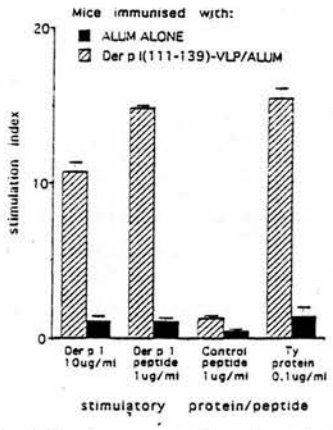

Fig. 4. Proliferative T cell responses to Der p 1 protein, Der p 1 (113–127) peptide, Ty protein and control I-A^b-binding peptide following immunization of C57BL/6 mice with Der p 1 (111–139)-VLP prepared in alum, or alum alone.

cells to Der p 1 (113–127) were also measured (Table 3). Although proliferation to Der p 1 (113–127) was not detected in mice immunized with VLP without alum, specific peptide-induced IFN-γ production was measured. When the VLP were injected with alum, strong T cell proliferation and high levels of IFN-γ were observed. A proportion of the IFN-γ production was due to the inflammatory action of alum. Der p 1-specific T cells primed by alum or non-adjuvanted Der p 1 (111–139)-VLP produced low levels of IL-5. IL-6 was only produced at low levels by T cells primed with alum adjuvanted VLP.

Discussion

Recent studies would tend to suggest that CD8⁺ T cells play a role in the resolution of the allergic disease process (17,18,29,30) but no MHC class I-restricted epitopes have so far been identified for any allergen. Using motif prediction methods successfully applied to defining viral CTL epitopes, we examined the Der p 1 protein sequence. One sequence, 111–119 (FGISNYCQI), conformed to the motif described for both K^b and D^b molecules (D^b motif, N at position 5 and I at position 9; K^b motif, Y at position 5 and I at position 8). Furthermore, the predicted CTL epitope (111–119) is overlapping but distinct from the CD4⁺ T_h epitope (113–127). The prediction of human class I-binding epitopes in allergenic proteins using defined motifs may lead to the identification of peptides which may be of therapeutic benefit.

In order to test whether the predicted MHC class I-restricted epitope is functional, in terms of its ability to prime CD8⁺ T cell responses, we produced recombinant hybrid VLP carrying Der p 1 (111–139). We had previously shown that hybrid VLP were potent inducers of CD8⁺ T cell responses when injected into mice without adjuvant (24,25) and immunization of C57BL/6 mice with Der p 1 (111–139)-VLP was indeed found to prime a strong Der p 1 (111–119)-specific

Table 3. Proliferative T cell and cytokine responses in draining lymph node cells following s.c. immunization with Der p 1 (111–139)-VLP (50 μg) with or without alum adjuvant

In vitro	*In vivo*			
	Der p 1 (111–139)-VLP in alum		Der p 1 (111–139)-VLP no adjuvant	
	p (113–127)[a]	Control[b]	p (113–127)[a]	Control[b]
Proliferation (SI)	7.3	1.0	1.0	1.0
IFN-γ (pg/ml)	5648.8	883.9	1403.2	<47
IL-5 (pg/ml)	180.2	33.8	251.9	51.7
IL-6 (pg/ml)	114.8	<20	<20	<20

[a]Der p 1 (113–127) peptide (10 μg/ml) was used to re-stimulate lymph node cells *in vitro* for 48 h (cytokines) or for 5 days (proliferation).
[b]Control wells represent unstimulated lymph node cells.

CTL response which was shown to be D^b-restricted. VLP carrying the minimal epitope (111–119) also primed strong CTL responses when injected by three different routes including intranasal. There was no detectable proliferative $CD4^+$ T_h cell response but a weak IFN-γ response to the overlapping I-A^b-restricted epitope (113–127) in the DLN of mice immunized with unadjuvanted Der p 1 (111–139)-VLP. This suggests that intracellular localization of the Ty p1–Der p 1 (111–139) fusion protein, following particle uptake by antigen-presenting cells, favours the generation of the MHC class I-binding peptide (111–119) but does not exclude the generation of MHC class II-binding (113–127) peptides. Evidence for the dual processing of the Der p 1 (111–139) sequence presented on VLP is supported by the greatly increased potency of Der p 1 (111–119)-VLP over Der p 1 (111–139)-VLP for CTL priming.

CTL activity was associated with very high levels of IFN-γ, low levels of IL-5 and no IL-6, and such a T_h1-type response may have a role in down-regulating IgE production to the allergen via suppression of IL-4 and IL-6 production (6,7). This has been observed in a rat model of respiratory allergy to ovalbumin in which tolerance to aerosolized ovalbumin can be adoptively transferred by IFN-γ-producing $CD8^+$-enriched splenocytes (17). These cells and similar suppressor cells in the mouse have been shown to express γδ TCR. It is currently not known what type of TCR (αβ or γδ) is expressed by $CD8^+$ T cells primed by VLP. Suppression of ovalbumin-specific IgE production and normalization of airways responsiveness was also seen in mice following the transfer of IFN-γ-producing $CD8^+$ T cells purified from the spleens of ovalbumin-sensitized mice (18). Recent studies in rats have shown that DNA immunization with another Der p allergen (Der p 5) results in the inhibition of allergen-induced IgE synthesis and airway hyper-responsiveness, and this effect is transferable with $CD8^+$ T cells (19). However, the allergen epitope specificity of the $CD8^+$ T cells is unconfirmed. IFN-γ also inhibits the production of IL-10, a T_h2 cytokine which down-regulates T_h1 responses (31).

The induction of T1-type $CD8^+$ T cells may also result in the production of other cytokines known to down-regulate T_h2/T2-type responses, e.g. IL-12 is known to inhibit T_h2-type and promote T_h1-type T cell responses probably via release of IFN-γ and also enhances the generation of $CD8^+$ T cells (32). Furthermore, it has been shown that CD30 ligand expressed on $CD8^+$ T cells may serve to inhibit Ig switching to IgE synthesis via interaction with CD30 on B cells (33). The apoptosis-inducing Fas ligand is also strongly expressed on activated $CD8^+$ T cells and can mediate the death of activated $CD4^+$ T cells (34). There are, therefore, several mechanisms whereby $CD8^+$ T cells may down-regulate T_h2-type T cell responses. However, recent studies have also shown that $CD8^+$ T cells were actually required for the development of airway responsiveness to ovalbumin in mice and that these cells produce IL-5 (35). Furthermore, $CD8^+$ T cells may be switched to produce T2-type cytokines if the cells are generated in the presence of IL-4 (36–38). It will be interesting to evaluate the effects of Der p 1 (111–139)- or (111–119)-VLP on mice experiencing an ongoing T_h2 response to Der p 1.

It is unclear, currently, whether the regulation of allergic responses in man is attributable to allergen-specific $CD8^+$ T cells and it is also unknown whether their cytotoxic effector function is important. However, mechanisms exist for the induction of MHC class I-restricted T cell responses by exogenous allergenic proteins, at least in rodents (39,40). It is also conceivable that *in vivo* administration of the Der p 1 (111–139) peptide, as previously described (11,12), or even Der p 1 (111–119) can induce Der p 1 (111–119)-specific $CD8^+$ T cells *in vivo* (41). We have previously shown that the intact particle is critical for CTL induction to epitopes presented on VLP (42), and that free peptides and soluble proteins at the same epitope molar equivalent as VLP do not prime CTL *in vivo* (24). It will be interesting to compare the immunogenicity of Der p 1-VLP with native Der p 1 protein and synthetic peptides for CTL and T_h cell priming.

As anticipated from previous studies (23), alum-adjuvanted VLP carrying Der p 1 (111–139) generated a strong (113–127)-specific $CD4^+$ T cell response in the DLN but did not prime $CD8^+$ CTL in either the lymph nodes or spleen. This is due to the denaturation of particles and the switching of uptake to the MHC class II processing pathway (42). Suprisingly, the alum-adjuvanted Der p 1 (111–139)-VLP primed a strong T_h1-type $CD4^+$ T cell response rather than T_h2. Alum is generally regarded as an adjuvant which predisposes to a T_h2-type responses in rodents (43). The nature of the antigen is, therefore, a critical factor in directing T cell responses towards a particular polarity. VLP carrying the minimal class

II-restricted epitope (113–127) also primed Der p 1-specific $CD4^+$ T cell responses (data not shown). The induction of Der p 1-specific $CD4^+$ T_{h1} cell responses in individuals experiencing an ongoing T_h2 cell response to this allergen may have a therapeutic benefit.

In summary, therefore, we have predicted a potential $CD8^+$ T cell epitope, residues 111-119, within the Der p 1 sequence 111–139 and have demonstrated a T1-type $CD8^+$ CTL response in C57BL/6 mice following one injection of VLP carrying either Der p 1 (111–139) or (111–119) without adjuvant by a variety of routes. The capacity to prime T_{h1}/T1-type $CD4^+$ and/or $CD8^+$ T cell responses to Der p 1 provides the means to study the regulatory role of these cells in models of HDM allergy in mice.

Acknowledgements

We thank all the members of the Virology/Immunology group at British Biotech. J. R. L. and G. F. H. were supported by the Wellcome Trust and the Medical Research Council.

Abbreviations

CTL	cytotoxic T lymphocyte
Der p 1	*Dermatophagoides pteronyssinus* 1
DLN	draining lymph node
HDM	house dust mite
INP	influenza virus nucleoprotein
VLP	virus-like particle
VSV	vesicular stomatitis virus

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Res. Immunol.
1998, 149, 235-240

Peptide-mediated regulation of allergen-specific immune responses

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Allergic inflammatory responses are regulated by cytokines derived from Th2-type helper cells. Therefore, the ability to downregulate the function of these T cells may contribute to the improvement of clinical symptoms. Two broadly based approaches to $CD4^+$ T-cell-targeted immunotherapy employing peptides are under consideration, namely the inhibition of Th2-cell activity and the induction of specific Th1 immunity. Evidence acquired from clinical trials using conventional allergen immunotherapy suggests that successful desensitization may be accompanied by the induction of T-cell anergy and/or a shift in the IFNγ to IL4 ratio in favour of the Th1 pathway.

Introduction

The primary aim of allergen immunotherapy (IT) is to vaccinate with allergen or its derivatives under conditions that will inhibit only the allergen-specific response, leaving the remainder of the immune system functionally intact. In addition to specific IgE, a hallmark of allergic inflammation is both the presence of $CD4^+$ T cells expressing a Th2-dominant cytokine profile [1, 2] and eosinophil infiltration at the disease site. Th0 cells with the potential to produce both Th2 (IL4, IL5, IL10 and IL13) and Th1 (IFNγ) cytokines also form a component of the T-cell repertoire in allergic inflammation. It is suggested that eosinophils contribute to airway hyperresponsiveness in asthma through epithelial damage by basic granule proteins, and that similar mechanisms may contribute to upper airway inflammation in rhinitis. Eosinophil chemoattractant chemokines, in particular eotaxin and RANTES, are now recognized as important factors leading to this eosinophil response. $CD4^+$ T cells are involved in regulating the synthesis of these chemokines, and through their capacity to produce IL5 and other cytokines, can facilitate the differentiation of inflammatory cells, including eosinophils. In subjects who are not genetically susceptible to allergic disease, T cells reactive with allergen can be identified in the peripheral repertoire but they are representative of the Th1 phenotype [3]. $CD4^+$ T cells are therefore central in immune responses to allergens as regards the regulation of allergic inflammation in allergic rhinitis and asthma, and the induction of "protective" immunity in normal individuals. As such, $CD4^+$ T cells provide an obvious target for immunotherapy. Evidence from independent clinical trials of conventional allergen desensitization indicate that successful immunotherapy may be accompanied by the induction of anergy in the peripheral T-cell repertoire and/or a shift in the balance of the IFNγ to

Received April 1, 1998.

IL4 ratio in favour of the Th1 pathway [4-7]. Although successful for a number of allergens, desensitization with intact native allergen carries the risk of inducing anaphylaxis and therefore the need to develop novel therapeutics remains.

There are two basic approaches to targeting the $CD4^+$ T cells, one is to render Th2-type cells or Th2-type cytokine production by Th0 cells of the appropriate specificity anergic/tolerant to allergen rechallenge (table I and reviewed in [8]). From clinical investigations on IT with bee venom, it appears that this functional state (i.e. anergy/tolerance) can be induced *in vivo* as a result of allergen desensitization [7]. There are a number of *in vivo* experimental animal models demonstrating that IT with proteins and peptides is mediated by the induction of T-cell tolerance [8-11]. The second approach is to immunize with peptides and select conditions of antigen presentation that will promote the induction of Th1 cytokines, one example of which is the use of recombinant mycobacteria expressing allergen-derived peptides [12].

Approaches that target surface proteins on a variety of different cell types, such as coreceptors (e.g. CD4), costimulatory receptors/ligands (e.g. CD28, CD80 and CD86) and adhesion molecules also offer potential ways of inhibiting allergic inflammatory responses. Similarly, antibodies capable of blocking IgE and mutated forms of cytokines (e.g. IL4 and IL5) and chemokines may have therapeutic applications in the regulation of allergic diseases. However, these approaches lack the unique specificity that may be possible to achieve through regulating subpopulations of CD4 T cells with specific antigen.

Table I. Approaches for peptide-mediated immune intervention.

1. Downregulate Th2 and/or redirect Th0 cells
   - **Allergen-derived peptides**
     Native sequence
     Altered T-cell ligands
2. Promote Th1 cells
   - **Vaccine vectors**
     Virus-like particle-allergen hybrid proteins
     Mycobacteria (*M. vaccae*, BCG)

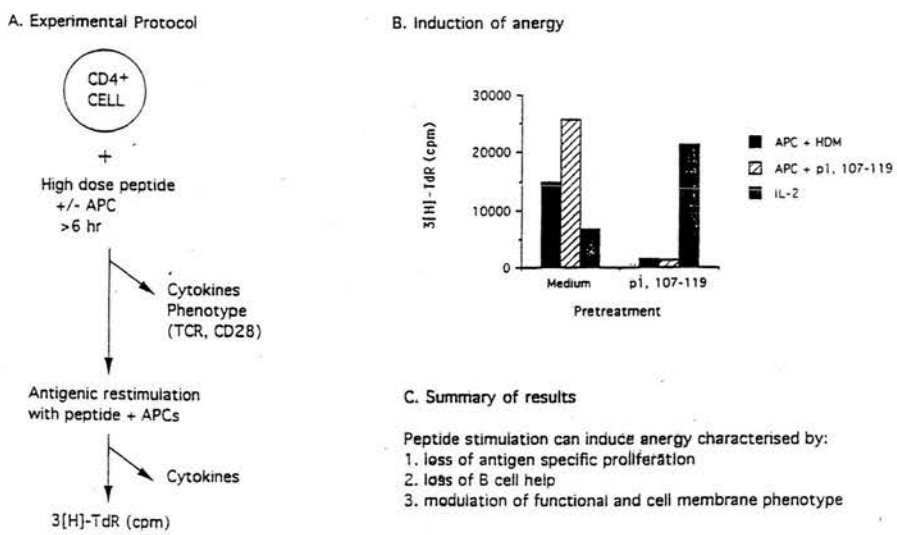

Fig. 1. *In vitro* modulation of allergen-specific human $CD4^+$ T cells.

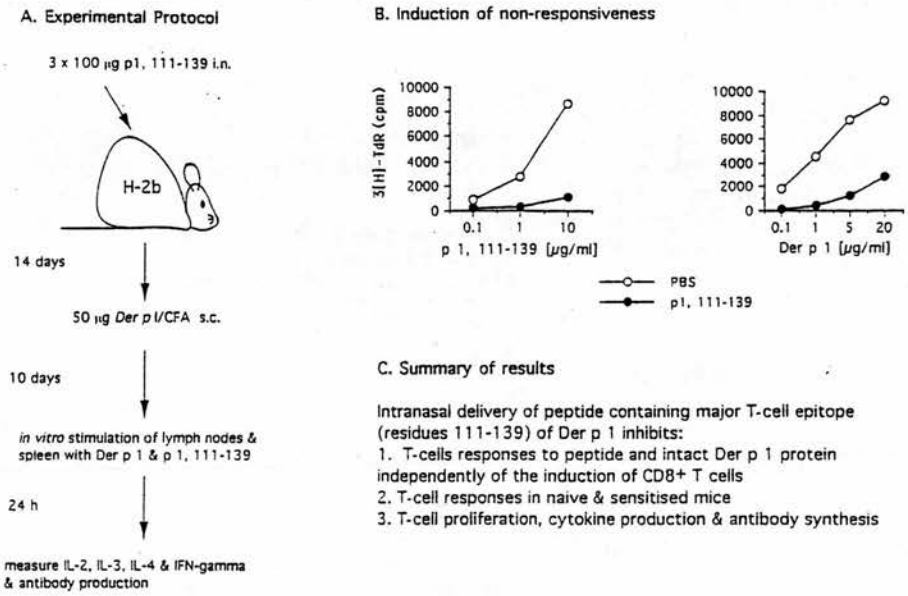

Fig. 2. *In vivo* modulation of murine responses to HDM-derived allergens following intranasal delivery of peptide.

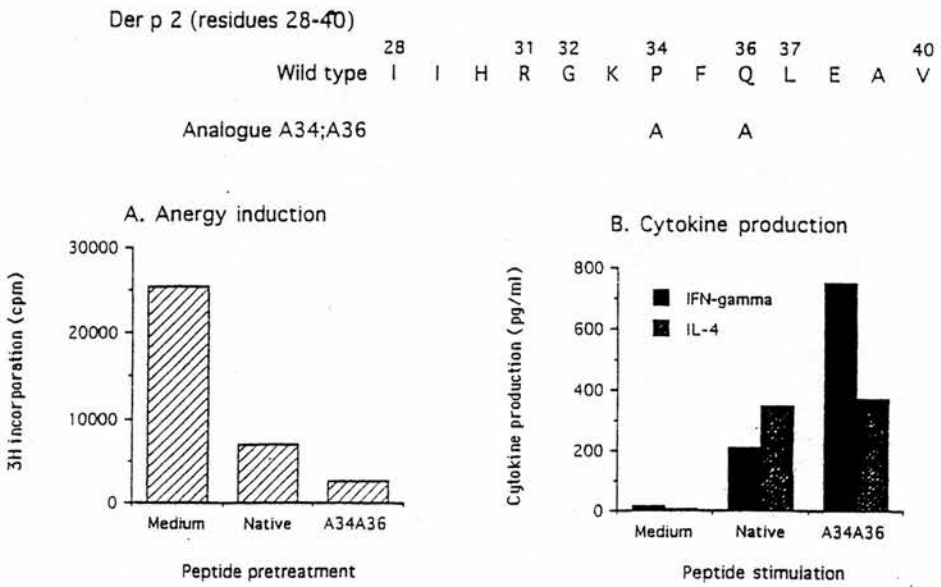

Fig. 3. Effect of peptide analogues on induction of T-cell anergy.

Allergen-derived peptide-mediated inhibition of Th2 cytokine production

The ability of peptide fragments of allergen to modulate the function of $CD4^+$ T cells has been demonstrated *in vitro* using human T cells ([13] and fig. 1) and in experimental animal models *in vivo* (ref. [8] and fig. 2). Information on peptide IT is now also becoming available from clinical trials in man and the results to date are variable [14-16].

In addition to their ability to induce T-cell unresponsiveness/tolerance, it has been reported that peptide ligands in which the T-cell receptor (TCR) contact residues have been modified by single amino acid substitutions, termed altered T-cell ligands [17], can modify the pattern of cytokine production by Th0 cells ([18] and fig. 3). The alteration in the affinity of the interaction between TCR and its ligand (MHC class II and peptide) appears to deliver an incomplete signal such that only selected effector function of the T cells is induced.

The immunological changes that occur in peptide mediated anergy/tolerance are summarized in table II.

Table II. Immunomodulation by allergen-derived peptides.

Native peptide

- Activation *in vitro* and *in vivo*
- Induction of antigen-specific unresponsiveness/tolerance

In vitro:
Loss of antigen-specific proliferation
Loss of B-cell help
Modulation of surface phenotype
(TCR↓, CD28↓, CD25↑, CD40L↔, CD80↔, CD86↔)
Induction phase transient activation
(cytokine-specific mRNA↑)
Modulation of cytokine production on restimulation
(IL4↓, IL5↓, IFNγ↔)
Induction of TGFβ

In vivo:
Transient activation
Inhibition of responses to all epitopes and intact protein independent of $CD8^+$ T cells
Inhibition of responses in sensitized and naive mice
Inhibition of proliferation, cytokine production and antibody synthesis
Repeated inhalation of peptide induces only very low levels of peptide-specific IgM, but not IgG2a or IgG1

Altered T-cell ligands

- Activation *in vitro*
- Induction of altered T-cell effector function
  - Dissociation of proliferation from IL4 production by Th2 cells
  - Inhibition of proliferation and cytokine production by Th0 cells
  - Enhanced production of IFNγ by Th0 cells
  - More effective induction of anergy

Virus-like particle-allergen hybrid proteins

Particulate antigen presentation based on the Ty p1 protein, which forms virus-like particles (VLPs) when expressed in yeast, is efficient in generating specific $CD4^+$ Th1-type responses when injected with the adjuvant alum and $CD8^+$ cytotoxic T-cell (CTL) responses in the absence of alum [19]. This has been demonstrated in human immunizing with VLP-gp120 constructs. In order to test this approach for the regulation of responses to allergen, H-2^b mice were immunized with VLPs expressing the immunodominant T-cell epitope of Der p 1 (residues 111-139), a major allergen derived from house dust mites (HDM). Immunized with p 1, 111-139-VLPs in alum induced Th1-type $CD4^+$ cells that responded when restimulated *in vitro* with either the appropriate Der p 1 peptide or the native protein. Furthermore, immunization with p 1, 111-139-VLPs without adjuvant induced a strong 111-119-specific D^b-restricted $CD8^+$ CTL response ([20] and fig. 4). The VLPs also induced a long-lasting CTL response following immunization by various routes. Both Der-p-1-specific CTLs and T helper cells produce high levels of IFNγ. The role of specific $CD8^+$ T cells in the regulation of allergic responses in man is unclear, although there are reports that the number of $CD8^+$ T cells in the periphery increases following desensitization.

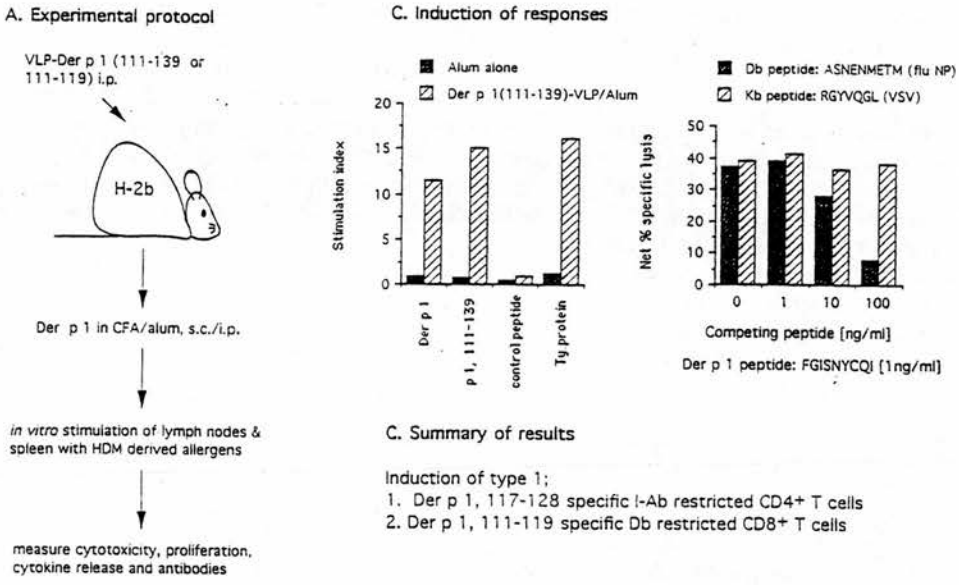

Fig. 4. Experimental animal model: immunization with Der p 1 (111-139)-VLPs.

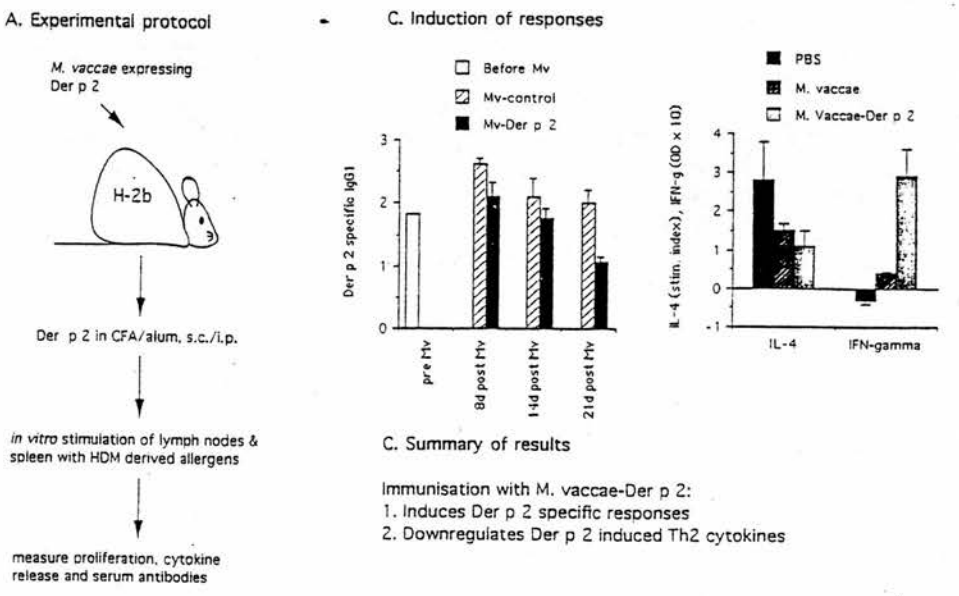

Fig. 5. Experimental animal model: immunization with *M. vaccae* expressing Der p 2.

Use of mycobacteria expressing peptides and proteins to HDM to "reprogram" specific Th2 immunity

Mycobacteria are known to be highly immunostimulatory, and through their uptake and presentation by macrophages favour the stimulation of Th1-type responses by the production of IL12 and IFNγ. Therefore, mycobacteria seemed an appropriate live vaccine vector for the expression and presentation of HDM genes for the induction of Th1 cells. Antigenic determinants from HDM proteins have been engineered into an extended loop of the superoxide dismutase (SOD) molecule of *M. tuberculosis*, and expressed in *M. vaccae*, a non-pathogenic species. Antigenic regions as large as 30 amino acids in length can be efficiently expressed in this way.

H-2^b mice were primed subcutaneously with heat-killed recombinant (r) *M. vaccae* expressing (SOD-p1) or without (SOD), the immunodominant peptide of the Der p 1 (residues 111-139). Immunization with r*M. vaccae* SOD-p1 induced recall responses to specific peptide that were predominantly of the Th1 phenotype, with little or no IL5 detectable. These experiments were extended and mice were injected with r*M. vaccae* SOD-p1 or r*M. vaccae* SOD and subsequently immunized with peptide to induce a specific Th2-dominant response. Enhanced levels of IFNγ together with a marked reduction in IL5 synthesis were observed in r*M. vaccae* SOD-p 1-immunized mice but not the r*M. vaccae* SOD or PBS control groups [12].

In another construct, the gene for the entire Der p 2 protein has been expressed in *M. vaccae* under the regulation of the 19-kDa promoter. Preliminary studies have shown that mice with an ongoing Th2-dominant response specific for Der p 2 exhibit an upregulation of of specific IFNγ *in vitro* following subcutaneous immunization with heat-killed *M. vaccae* expressing intact Der p 2 (fig. 5). In addition, in these mice, the decline in IgG1 antibodies was more rapid than in control mice.

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INFECTION AND IMMUNITY, Aug. 1998, p. 3643–3648
0019-9567/98/$04.00+0

Vol. 66, No. 8

An Epitope Delivery System for Use with Recombinant Mycobacteria

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Received 29 December 1997/Returned for modification 5 March 1998/Accepted 12 May 1998

We have developed a novel epitope delivery system based on the insertion of peptides within a permissive loop of a bacterial superoxide dismutase molecule. This system allowed high-level expression of heterologous peptides in two mycobacterial vaccine strains, *Mycobacterium bovis* bacille Calmette-Guérin (BCG) and *Mycobacterium vaccae*. The broader application of the system was analyzed by preparation of constructs containing peptide epitopes from a range of infectious agents and allergens. We report detailed characterization of the immunogenicity of one such construct, in which an epitope from the Der p1 house dust mite allergen was expressed in *M. vaccae*. The construct was able to stimulate T-cell hybridomas specific for Der p1, and it induced peptide-specific gamma interferon responses when used to immunize naive mice. This novel expression system demonstrates new possibilities for the use of mycobacteria as vaccine delivery vehicles.

Mycobacteria are potent immunogens, inducing both antibody and cell-mediated immune responses. BCG, an attenuated strain of *Mycobacterium bovis*, is the most widely used vaccine worldwide; *Mycobacterium tuberculosis* is routinely used for preparation of complete Freund's adjuvant; and components of the mycobacterial cell wall have been exploited in development of alternative adjuvant systems (4, 13, 23). With the advent of tools for genetic manipulation of mycobacteria, several reports have described the use of recombinant BCG as a potential vaccine vector (2, 19, 31, 32). A strain of BCG expressing the *ospA* antigen of *Borrelia burgdorferi* has been shown to induce protective immunity in animal models, for example (31).

With the aim of understanding factors underlying the immunogenicity of mycobacteria, and the eventual construction of improved mycobacterial vaccines, we wished to analyze the ability of mycobacteria to present a series of well-defined antigens to the immune system in experimental models. We have chosen initially to compare the immunogenicity of BCG, delivered as a live vaccine, with that of *Mycobacterium vaccae*, a soil organism that has been used as a killed preparation in human immunotherapy (9, 30). In initial experiments with a range of antigens, we were able to obtain only low-level expression and observed a tendency for decreased expression during subculture of recombinants. To obtain a high-level expression system suitable for use with different antigens, we have developed an epitope expression system based on the iron-containing superoxide dismutase (SOD) of *M. tuberculosis*.

M. tuberculosis SOD is a multimeric protein formed by the tight association of four 23-kDa subunits. SOD is located predominantly in the cytoplasm of mycobacteria but is also found in the extracellular fluid of cultures of slowly growing mycobacteria such as *M. tuberculosis* (3) and *Mycobacterium avium* (8). It was initially identified as a major target of the immune response in mice immunized with mycobacteria. The gene encoding the *M. tuberculosis* enzyme can be expressed at high levels in recombinant mycobacterial systems, accounting for as much as 10% of the total cell protein (11). High-level expression in *M. vaccae* has been exploited for protein purification, allowing establishment of the complete three-dimensional structure of the protein (6). Analysis of this structure identified an outward-extending loop which is distal from the reactive site of the enzyme and is not involved in subunit interactions. In the present study, we describe a strategy for epitope expression in mycobacteria which is based on targeting of this loop as an insertion site for foreign peptides.

MATERIALS AND METHODS

Epitope carrier vector construction. A *Bam*HI site was created in the *sodA* gene, present on plasmid p16R1/SOD (11), using two-step PCR-directed mutagenesis. The resulting plasmid, designated p23.1, contains a unique *Bam*HI site between codons 51 and 53 of the *sodA* gene. Complementary oligonucleotides flanked by *Bam*HI sites were subsequently used to introduce immunodominant peptides from various antigens (Table 1). In each case, codon usage was optimized for that of known *M. tuberculosis* proteins. Constructs were routinely checked by DNA sequence analysis.

Bacterial cultures and transformation. *Escherichia coli* DH5α was grown at 37°C in LB medium. *M. vaccae* NCTC 11659 (supplied by John Stanford, University College and Middlesex Hospital School of Medicine, London, United Kingdom) was grown in Middlebrook 7H9 medium supplemented with 2% glucose. *M. bovis* BCG (strain P3) was grown in Middlebrook 7H9 medium supplemented with albumin, dextrose, and catalase as recommended by the manufacturer (Difco, West Molesey, United Kingdom). When appropriate, hygromycin B (Sigma, Northampton, United Kingdom) was added at 200 μg/ml for *E. coli* or at 50 μg/ml for *M. vaccae* and BCG (13). Transformation of mycobacteria was carried out by electroporation (11), whereas *E. coli* was transformed by using standard procedures (27). For immunization purposes, *M. vaccae* recombinants expressing chimeric SOD proteins were grown on 7H11 plates supplemented with 2% glucose and 50 μg of hygromycin per ml.

Analysis of expression of chimeric SOD proteins. For analysis of protein expression, sonicated extracts of recombinant *M. vaccae* or BCG were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) (22) and Western blotting. Blots were developed with a monoclonal antibody (D2D) against mycobacterial SOD (34) and visualized by chemiluminescence (Amersham International, Amersham, United Kingdom). The en-

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TABLE 1. Constructs used

Construct	Peptide sequence	Origin	Reference(s)	Length (residues)	Expression
SOD					+
SOD-p1	111–139 of Der p1	House dust mite	16, 20	29	+
SOD-p2	87–105 of Der p2	House dust mite	17	14	++
SOD-OVA	323–339 of ovalbumin		29	17	++
SOD-HA	126–138 of hemagglutinin	Influenza virus	28	22	+++
SOD-NP	366–374 of nucleoprotein	Influenza virus	33	9	+++
SOD-M2	58–66 of matrix protein	Influenza virus	1	9	+++
SOD-48	307–319 of hemagglutinin	Influenza virus	26	13	++
SOD-3.20	335–349 of nucleoprotein	Influenza virus	33	15	++
SOD-45.1	380–391 of nucleoprotein	Influenza virus	18	12	++
SOD-65	1–20 of Hsp65	*M. tuberculosis*	12	20	+
SOD-40.6	Various epitopes[a]	Plasmodium	15	30	+
SOD-36.8	Various epitopes[b]	Plasmodium	15	60	±
SOD-RSV	82–90 of 22-kDa protein	Respiratory syncytial virus	21	9	+++

[a] CD8 epitopes of STARP and LSA-1 and a CD4 epitope of CS protein (15).
[b] CD8 epitopes of STARP, TRAP, CS protein, and LSA-1 and a CD4 epitope of CS protein (15).

zymatic activity of recombinant SOD proteins was examined by activity staining of gels run under nondenaturing conditions (36).

Preparation of antigens. Aliquots of 100 mg/ml of recombinant mycobacterial suspensions were frozen at −70°C in 15% glycerol. For immunization, the glycerol was washed away, the bacteria were heat-killed (20 min, 80°C) where necessary, and the preparations were resuspended at the appropriate concentration in phosphate-buffered saline–0.05% Tween 80. Soluble native *M. vaccae* antigens for in vitro stimulation were prepared by sonicating the bacterial suspensions in phosphate-buffered saline and filtering them through a 0.22-μm-pore-size filter. Peptide 111–139 of Der p1 (sequence, FGISNYCQIYPPNANK IREALAQTHSAIA [5]) and peptide 111–119 were made on an ABIMED 422 synthesizer (ABIMED, Langenfeld, Germany) by the simultaneous peptide synthesis method. The purity of the peptide was verified by reverse-phase C_{18} high-pressure liquid chromatography (Lichrospher; Merck, Darmstadt, Germany) and was shown to be routinely over 75%.

Animals and immunization protocols. Female C57BL/6J (*H-2*^{b}) mice (6 to 8 weeks old) from Harlan-Olac (Bicester, United Kingdom) were used in the study. Mice, usually in groups of four, were immunized subcutaneously (s.c.) in the flank with preparations of 1 to 1,000 μg of *M. vaccae* (corresponding to 10^6 to 10^9 organisms) in a 200-μl volume.

Cell culture. Single-cell suspensions of splenocytes or lymph node cells were cultured in 96-flat-well microtiter plates (Nunc, Roskilde, Denmark) at 2×10^6 cells/ml in RPMI 1640 supplemented with 5% fetal calf serum, 2 mM L-glutamine, 20 IU of penicillin and 20 μg of streptomycin (Gibco, Grand Island, N.Y.) per ml, and 0.05 μM 2-mercaptoethanol (Sigma, St. Louis, Mo.) (200 μl/well) at 37°C in humidified air containing 5% CO_2. Cells were incubated in triplicate wells alone or with various concentrations of antigens for the assessment of gamma interferon (IFN-γ) in supernatants at 72 h. Proliferation was measured by pulsing for the last 6 h with [^{3}H]thymidine (0.5 μCi/well; Amersham), harvesting the contents of each well onto glass fiber mats, and determining the incorporation of ^{3}H in a Betaplate liquid scintillation counter (Wallac, Turku, Finland).

The isolation of Der p1-specific T-cell hybridomas has been previously described (20). Briefly, the hybridomas were generated by polyethylene glycol (Sigma)-induced fusion of Der p1-specific CD4$^+$ T-cell lines derived from *H-2*^{b} mice with the BW 5147 fusion partner (a kind gift from H. Bodmer, Nuffield Department of Clinical Medicine, Oxford, United Kingdom) and selection with hypoxanthine-aminopterin-thymidine medium (Sigma). Hybridomas recognizing epitopes between residues 111 and 139 of Der p1 were used in this study. Activation of the hybridomas was measured by culturing them at 5×10^4 cells/well with appropriate antigens and 10^5 irradiated (2,500 rads) syngeneic splenocytes as antigen-presenting cells (APC) for 20 h and assaying the interleukin-2 (IL-2) content of the culture supernatants.

Cytokine assays. IFN-γ was measured by enzyme-linked immunosorbent (ELISA) using the rat anti-mouse coating antibody R4-6A2 and biotinylated detector antibody XMG1.2 pair (PharMingen, San Diego, Calif.). The binding of biotinylated antibody was detected with alkaline phosphatase-conjugated streptavidin (Amersham) followed by *p*-nitrophenylphosphate (Sigma) at 1 mg/ml in Tris HCl buffer (pH 9.6) as the substrate. Optical density at 405 nm of the product was measured with a Bio-rad ELISA reader. Recombinant murine IFN-γ (PharMingen) was used to construct a standard curve.

IL-2 was measured in supernatants from the IL-2-dependent cell line CTLL-2. Briefly, 5×10^3 CTLL-2 (American Type Culture Collection) cells in 50 μl were incubated with 50 μl of test supernatant in triplicate for 24 h, and proliferation was measured over the last 6 h by pulsing with [^{3}H]thymidine (0.5 μCi/well; Amersham), harvesting the contents of each well onto glass fiber mats, and determining the incorporation of ^{3}H in a Betaplate liquid scintillation counter (Wallac).

ELISA for specific antibodies. Der p1 peptide 111–139 (5 μg/ml) was coated onto Maxisorp microtiter plates (Nunc) in bicarbonate coating buffer (Sigma) overnight at 4°C. After blocking (1 h, Tris-buffered saline–1% bovine serum albumin) and washing, serum dilutions were incubated in duplicate for 2 h at 37°C. After washing, the amount of specific immunoglobulin (IgG) isotypes bound was detected with alkaline phosphatase-conjugated rat anti-mouse IgG1 and IgG2a (PharMingen) for 1 h at 37°C. The enzyme substrate *p*-nitrophenylphosphate was added, and the soluble product was measured as described for the cytokine ELISAs.

Isolation of CD4$^+$ and CD8$^+$ cells. CD4$^+$ and CD8$^+$ T cells were isolated from lymph node cells from immunized mice by immunomagnetic selection. After incubation with biotinylated anti-CD4 (GK1.5; PharMingen) or anti-CD8 (clone SK1; Becton Dickinson, Oxford, United Kingdom), cells were incubated with streptavidin-conjugated microbeads (Miltenyi Biotec) (25) and selected by using the MACS system (Miltenyi Biotec). Positively isolated fractions were collected, and the efficiency of the separation was cross-checked by staining with rat anti-mouse CD4 conjugated to phycoerythrin (CT-CD4; Caltag, Burlingame, Calif.), rat anti-mouse CD8 conjugated to phycoerythrin (clone 53-6.7; Sigma), or fluorescein isothiocyanate-conjugated streptavidin (Amersham). Samples containing 10^5 cells from each of the stained fractions were analyzed on a Becton Dickinson FACScan flow cytometer.

RESULTS

Construction and expression of chimeric SOD proteins. To exploit the adjuvant activities of mycobacteria, a mycobacterial antigen delivery system for high-level expression of epitopes was developed by using the tetrameric SOD protein of *M. tuberculosis* as a carrier. To incorporate short peptide sequences into SOD, we targeted an outward-extending loop (positions 52 to 59 [Fig. 1]) between two alpha helices in the N-terminal domain (7) for several reasons. First, this domain does not seem to be involved in the enzymatic activity or in the interaction between the SOD monomers; second, the region around amino acid 53 appears to be particularly variable between different mycobacterial species, suggesting that variation in amino acid sequence in this region does not influence the structural and functional properties of the SOD molecule. We constructed a plasmid (p23.1) in which a unique *Bam*HI site was introduced between codons 51 and 53 of *M. tuberculosis* SOD. Expression of SOD was under the control of its native *M. tuberculosis* promoter, and the plasmid contained a mycobacterial origin of replication. This vector was subsequently used for the insertion of a variety of peptides derived from heterologous antigens listed in Table 1.

Constructs were introduced into *M. vaccae* or BCG, and expression was monitored by Coomassie brilliant blue staining,

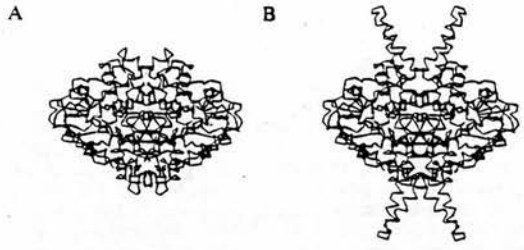

FIG. 1. Identification of a permissive loop in *M. tuberculosis* SOD. Inspection of the crystal structure of the tetrameric *M. tuberculosis* SOD (A) identifies an outward-extending loop centered on amino acid residue 53. For epitope expression, this region of the gene was engineered to contain a *Bam*HI site. Oligonucleotides encoding appropriate epitopes were inserted at this site to construct a series of chimeric SOD molecules. The structure of the one of the chimeric SOD proteins, SOD-HA, containing peptide 126–138 of influenza virus HA, was predicted via computer modelling (B) using the known structure of SOD and the known structure of HA. The modelling was done with QUANTA (Molecular Simulations Incorporated), using conventional loop-database search to find homologous fragments with the appropriate distance-geometry to fit the framework of the protein. The core SOD protein (Fig. 1A) is also present in the predicted chimeric SOD-HA protein (Fig. 1B), and the inserted epitope is in an extended loop far from functional sites of the protein.

Western blotting, and enzyme activity. Figure 2A illustrates expression of two chimeric SOD proteins in *M. vaccae* extracts stained for total protein. The chimeric SOD proteins are among the most prominent bands in the extracts, although the expression levels are lower than that of the wild-type SOD protein (lane 1). Densitometric scanning of gels after SDS-PAGE indicated that the bands representing the chimeric SOD proteins correspond to 2 to 10% of the total protein in *M. vaccae* extracts.

The level of expression of recombinant proteins in BCG was lower than that in *M. vaccae* and was visualized by Western blot analysis using a monoclonal antibody specific for SOD. Figures 2B and C compare the level of expression of control SOD and three chimeric constructs in BCG and in *M. vaccae*. In each case, the amount of recombinant protein was approximately fivefold higher in *M. vaccae*. The 23-kDa protein seen in all lanes of Fig. 2B and C corresponds to the native SOD enzyme present in BCG and in *M. vaccae*, respectively. Intro-

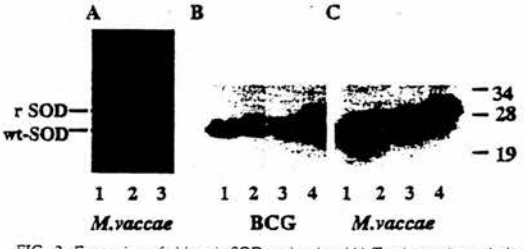

FIG. 2. Expression of chimeric SOD molecules. (A) Total protein analysis. Sonicates from *M. vaccae* recombinants expressing wild-type SOD (wt-SOD; lane 1), SOD-p1 (lane 2), and SOD-p2 (lane 3) were fractionated by SDS-PAGE, and the gel was stained with Coomassie brilliant blue. Recombinant (r) SOD proteins are seen as major bands. (B and C) Western blot analysis. Extracts from BCG (B) and *M. vaccae* recombinants (C) were separated by SDS-PAGE, transferred to nitrocellulose, and stained with a monoclonal antibody to SOD. Lane 1, wild-type SOD; lane 2, SOD-p1; lane 3, SOD-p2; lane 4, SOD-HA. The endogenous SOD from BCG and *M. vaccae* SOD is seen in all lanes (lower band). Sizes are indicated in kilodaltons.

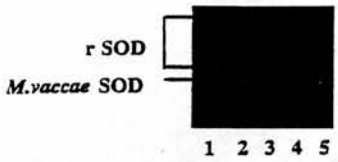

FIG. 3. SOD activity in nondenaturing gels. Sonicates from *M. vaccae* recombinants were run on nondenaturing gels and stained for SOD enzyme activity. Native SOD activity from *M. vaccae* is seen as the two lower bands present in all lanes. The variable upper bands represent a series of hybrid tetramers formed by the recombinant (r) SOD. Lane 1, wild-type SOD; lane 2, SOD-OVA; lane 3, SOD-p1; lane 4, SOD-p2; lane 5, SOD-HA.

duction of the same constructs into another rapidly growing mycobacterium, the laboratory strain of *M. smegmatis*, resulted in levels of expression similar to those observed in BCG (data not shown).

Assay of SOD activity in nondenaturing gels revealed that the chimeric proteins were expressed as functional enzymes (Fig. 3). As reported previously (35), expression of the wild-type *M. tuberculosis* SOD in *M. vaccae* generated the *M. tuberculosis* tetramer (the predominant top band seen in lane 1) as well as a series of hybrid enzymes containing different combinations of *M. tuberculosis* and *M. vaccae* subunits. A similar pattern was seen with the chimeric proteins, although differences in size and charge led to altered mobility of the tetramers during gel electrophoresis. These data suggest that the incorporation of epitopes in the extended loop of SOD is tolerated without having a major effect on enzyme activity or on the ability to form tetramers. The structure of one of the SOD chimera was predicted by computer modelling. Since the crystal structure of hemagglutinin (HA) of influenza virus is known, the SOD-HA protein was chosen. Consistent with the experimental data, comparison of the known structure of SOD (Fig. 1A) with the predicted structure of SOD-HA (Fig. 1B) revealed that the folding of the active SOD core of the SOD-HA protein is identical to that of the wild-type protein.

Antigenicity of the recombinant *M. vaccae*. Since mycobacteria are potent inducers of cell-mediated immunity, we wished to study T-cell responses to epitopes delivered by mycobacteria as chimeric SOD proteins. Therefore, we focused on a construct that contains an immunodominant region from Der p1 (residues 111 to 139), one of the major allergens of house dust mites, which encompasses well-characterized murine major histocompatibility complex (MHC) class I- and class II-restricted T-cell epitopes (residues 111 to 119 and 113 to 127, respectively) (14, 16, 17, 20). The antigenicity of the chimeric SOD protein containing Der p1 (111–139) expressed in *M. vaccae* (Mv-p1) was examined by measuring its ability to stimulate two different murine $CD4^+$ T-cell hybridomas recognizing epitopes within the region from residues 111 to 139 of Der p1. Both hybridoma 1BB8 (Fig. 4) and hybridoma AD2 (results not shown) were activated by the presence of intact Mv-p1 and, to an even greater extent, by sonicated Mv-p1. In contrast, preparations of control *M. vaccae* expressing wild-type SOD (Mv-SOD) failed to stimulate any IL-2 production. These findings confirmed that the peptide expressed by Mv-p1 was in a form that could be recognized by peptide 111–139-specific murine T cells. Comparison of the response to sonicated Mv-p1 and to peptide and estimation of the amount of peptide in such a sonicate revealed that the antigenicity of a peptide delivered by *M. vaccae* is similar to that of the peptide itself.

Immunogenicity of recombinant *M. vaccae*. The immunogenicity of Mv-p1 was tested by s.c. immunization of C57BL/6J

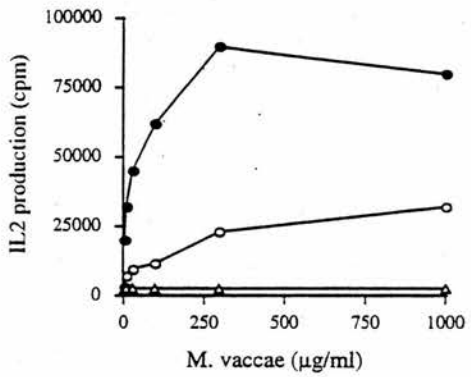

FIG. 4. Recognition of *M. vaccae* recombinants by T-cell hybridoma. Murine T-cell hybridoma 1BB8 specific for Der p1(111–139) was cultured in the presence of APC with sonicated or intact heat-killed Mv-p1 (filled and open circles, respectively) and sonicated Mv-SOD (open triangles), and supernatants were tested for IL-2, using the CTTL-2 cell line. Only the recombinant expressing the specific peptide stimulated the cells. For comparison, supernatant from the same hybridoma stimulated with 0.1 μM synthetic peptide produced 93×10^3 cpm in the IL-2 assay.

mice with 5×10^8 (500 μg) heat-killed Mv-p1 bacilli without adjuvant. A heat-killed preparation was used for two reasons. First, heat-killed *M. vaccae* is clinically applicable in humans, and second, *M. vaccae* is not believed to survive after immunization, suggesting no extra benefit for the use of viable bacteria. Five days later, draining lymph nodes were isolated and T-cell responses were evaluated by measuring IFN-γ production and proliferation in response to in vitro stimulation with peptide 111–139. Immunization with Mv-p1 resulted in the production of high levels of peptide-specific IFN-γ by lymph node cells in culture (Fig. 5A) and peptide-specific proliferation (data not shown). This finding demonstrates that the peptide is immunogenic when presented in vivo as an SOD chimera delivered by *M. vaccae*. A control immunization with 5×10^8 heat-killed Mv-SOD did not result in peptide-specific IFN-γ responses. However, when a soluble extract of *M. vaccae* was used as an antigen, an equally strong IFN-γ response was observed in mice immunized with either Mv-p1 or Mv-SOD.

Peptide 111–139 of Der p1 can also be recognized by B cells. Therefore, mice were immunized twice with a 3-week interval with 5×10^8 heat-killed or Mv-p1 or with control Mv-SOD. Serum samples were collected 10 days after boost and tested for the presence of peptide 111–139-specific IgG1 and IgG2a. No IgG1 was detectable. IgG2a titers between 30 and 100 were measured in mice that received Mv-p1. No peptide-specific antibodies were detectable in sera derived from control immunized mice. This finding indicates that it is possible to induce antibody responses by using the mycobacterial system for the delivery of this particular peptide, although this induction is very inefficient.

$CD4^+$ versus $CD8^+$ T cells as the source of IFN-γ. Since peptide 111–139 of Der p1 contains both a CD4 and a CD8 epitope, the cell type responsible for peptide-induced IFN-γ production was studied in more detail. CD4- and CD8-enriched cell populations were prepared by immunomagnetic fractionation of lymph node cells from mice inoculated 5 days previously with 5×10^8 heat-killed Mv-p1. By flow cytometry, the proportion of $CD4^+$ cells in the $CD8^+$ fraction and the proportion of $CD8^+$ cells in the $CD4^+$ fraction were routinely less than 2%. As shown in Fig. 5B, culture with Der p1 peptide resulted in production of IFN-γ by unfractionated lymph node cells and by $CD4^+$ cells (in the presence of syngeneic APC). No IFN-γ was produced by $CD8^+$ cells incubated with Der p1 peptide 111–139 or with peptide 111–119, which encompasses the CD8 epitope.

DISCUSSION

The expression of foreign antigens in recombinant mycobacteria is attractive as a strategy for vaccine production and more generally as an approach to probing mycobacterial interactions with the immune system. The fact that exogenous proteins are often expressed at low levels in mycobacteria imposes an important limitation on development of this approach. We have explored a peptide expression system in order to increase the

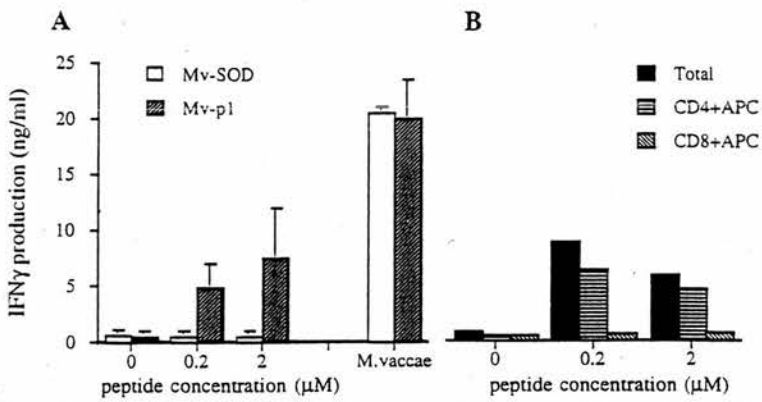

FIG. 5. Immunogenicity of *M. vaccae* recombinants. Mice were immunized s.c. once with Mv-p1 or Mv-SOD, and antigen-specific cytokine responses were monitored 5 days later in inguinal lymph node cells. (A) Lymph node cells (4×10^5 cells/well) from mice immunized with Mv-p1 produced IFN-γ when incubated with specific peptide or with *M. vaccae* extract (2.5 μg protein/well). Cells from mice immunized with control Mv-SOD responded only to the *M. vaccae* antigen. (B) Lymph node cells were fractionated into $CD4^+$ and $CD8^+$ populations and incubated (2×10^5 cells/well plus 3×10^5 irradiated APC/well) in the presence or absence of specific peptide. IFN-γ production was observed only with the $CD4^+$ population.

repertoire of epitopes that can be delivered by mycobacteria. We selected a native mycobacterial protein, SOD from *M. tuberculosis*, as a carrier protein based on its high level of expression and known crystal structure. While differences were observed in the level of expression of different peptides (Table 1), the SOD expression system was found to be broadly applicable to a wide range of epitopes, and we have been able to express chimeric SOD proteins containing as many as 60 amino acids. The success of this expression system is probably due to the inherent high level of expression and stability of the SOD molecule and the fact that the point of peptide insertion does not significantly disrupt assembly of the functionally active tetrameric enzyme. Expression of all tested chimeric proteins was higher in *M. vaccae* than in BCG, an effect that could be due to differences in promoter activity, plasmid copy number, or proteolytic activity between the two strains. In addition, expression of the endogenous *M. vaccae* and BCG SOD proteins in the two strains might interfere with expression of the plasmid-encoded chimeric SOD to differing extents.

A construct in which an immunodominant peptide from a major house dust mite allergen (Der p1) was expressed as a chimeric SOD protein in *M. vaccae* was selected for detailed immunological characterization. Intact or sonicated bacteria were recognized by murine T-cell hybridomas specific for the peptide, demonstrating that the chimeric SOD is available for antigen processing and presentation. More efficient recognition of sonicated extracts suggests that there may be differences in processing between the intact and disintegrated mycobacteria. These differences could be related to an inability of irradiated spleen cells to process intact mycobacteria, or perhaps to a difference in kinetics of processing. Immunization experiments demonstrated that recombinant mycobacteria were also able to induce a specific T-cell response to the Der p1 peptide in vivo, as assessed by IFN-γ production by lymph node cells. Since peptide 111–139 contains both a CD4 and a CD8 epitope (14), T-cell separation experiments were performed to determine the phenotype of the responding cells. These experiments, together with cytotoxicity assays (not shown), demonstrated the response to be exclusively attributable to $CD4^+$ T cells, suggesting that antigen presentation occurs only via MHC class II molecules.

Experiments with transgenic mice lacking the β2-microglobulin gene have demonstrated that T-cell responses restricted by MHC class I molecules play an important role in protection against *M. tuberculosis* infection (10), and recent in vitro experiments suggest that mycobacterial species differ in the ability to deliver antigens by this route (24). An important application of the expression system described here is that it will allow us to compare the ability of different mycobacteria to deliver the same antigen to the immune system. We are currently evaluating the immunogenicity of the recombinant BCG constructs with a view to comparison with *M. vaccae* and in some cases with virulent *M. tuberculosis*. This strategy provides an approach to identifying microbial factors involved in immunogenicity and may also allow us to select vaccine candidates with particular immunological profiles. We are also using a peptide approach to analyze the immunogenicity of an identical epitope expressed in a cytoplasmic (SOD) compared to a membrane-associated carrier protein in mycobacteria. Another application of this mycobacterial expression system is the use of such recombinants for immunomodulation. As mycobacteria are especially capable of inducing Th1 cell development, characterized by the production of high levels of IFN-γ and IL-2, it may be possible to use constructs expressing epitopes of allergens for modulation of allergen-specific Th2 cell responses, characterized by the production of high levels of IL-4 and IL-5. However, for this purpose the exact nature of T-cell responses raised with recombinant mycobacteria expressing epitopes of allergens has to be evaluated in more detail.

Diversity in the repertoire of peptides presented by different MHC molecules represents an obvious limitation to an epitope-based approach to therapeutic vaccine development. In the SOD expression system, it may be possible to address this problem in part by insertion of multiple epitopes within a single chimeric construct. The repertoire could be expanded further by expressing tetrameric proteins comprised of different recombinant monomers. For broader application, the epitope system could be used for initial experimental identification of a mycobacterial host strain with appropriate immunogenic properties, with a view to subsequent optimization of specific strategies for stable expression of each whole antigen molecule.

ACKNOWLEDGMENTS

We thank the EC, the Royal Society, the MRC, the Wellcome Trust, and the British Lung Foundation for financial support.

C. Hetzel and R. Janssen made equivalent contributions to this study.

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Editor: R. N. Moore